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EVOLUTION TOWARD A MATURE SCIENTIFIC LITERATURE¹

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I wish to thank the Society for a delightful memory and for a great honor. Nineteen years ago our former Secretary, Dr. Hitchens, officially sanctioned my first appearance before a national society and then took me to a café in old Philadelphia where I enjoyed the conversation of several famous men. That reception to the fellowship of scientific workers is typical of the fine spirit with which this Society has always welcomed its youngsters. For the great honor I owe more than I can express. There are aspects of its bestowal which I cannot mention without seeming to question your judgment; but I believe you will recognize my meaning when I say that its bestowal is evidence of the remarkable catholicity of this Society.

The discussions at our annual meetings broaden and deepen with the ever enlarging stream of scientific thought. This is inevitable since the study of life in any of its varied forms carries concerted thought of many kinds to ever harder tasks. It is a fact that in the lines of our printed programs, which describe investigations specific to our own science, our members write:—the languages of pathology and thermodynamics; the data of statistical and chemical analyses; the signs of potentiometric and optical measurements; the notations of enzymatic and genetic specificities; the symbols of chemical and cytological morphologies; names from the manuals of instrumentation and the catalogues of taxonomy. The contributions of morphologists, of physiologists, of chemists, of physicists, of mathematicians, of

¹ Presidential address delivered before the Society of American Bacteriologists at its Thirty-fifth Annual Meeting, Philadelphia, Pennsylvania, December 28, 1933.

those who are skilful in the numerous subsidiary subjects and in their recombinations to form the specialities and the contributions of those who deal with what may be called the special dimensions of biology are not of equal importance in the individual case but all are coördinated toward the comprehension of what, for lack of a better term, we call life. United within this broad meaning of biology the members of this Society come here from chairs and academies of theoretical learning and from institutes and field camps established to explore the frontiers of medicine and public health, the fields of agriculture and the provinces of industry. They come here to witness the confluence of many different streams of thought in solving problems of proven importance to the welfare of mankind or problems of importance to the whole of science, problems for which our specific science offers unique material. In such a comradeship there can be none of the confining jealousies of the old professional guilds but rather the recognition that it is the necessity for skill with the special tools of hand and mind that makes one of us a physiologist, another a taxonomist, another an immunologist. Indeed for the purposes of our specific science we would welcome within our fold ever wider interests, recognizing that those who huddle into narrow groups have pitifully failed to appreciate the great enlargement of those intellectual coöperations which would hold us had we no formal organization other than that which oils a simple machinery of meeting and of publication.

With the growing complexity of our science there grow not only those specific problems which must be solved by the labor and the insight of individuals but also problems of intellectual adjustment requiring the coöperation of all. A pressing problem of the second type arises from that necessity for breadth of knowledge which every student of life must feel and the tokens of which I have just noted. By these same tokens of our great desire and pressing need it is such as we who can judge, perhaps better than the devotees of some abstract science, the state of scientific literature. Of the scientific literature men have spoken in private with such despair that I feel compelled to raise their hopes by reviewing corrective and constructive forces displayed in history.

To do this with the frankness that the case demands I must betray a discord between my own words and deeds. I shall hang myself, but speak I must of crimes against the common weal.

To appreciate the forces which have been operating throughout the twentieth century let us look back to the first third of this century. Place yourself at the close of that period—1933—a date which now seems long ago and imagine that you were then a student of life.

For information upon current work you would have groped your way through about 30,000 yearly entries in Biological Abstracts, 37,000 yearly entries in Chemical Abstracts, and the 1269 pages of Quarterly Cumulative Index Medicus which itemized the articles in 1388 periodicals. It goes without saying that you would have ignored the greater part of these abstracts. Yet the hawk's eye was needed since quarry was to be found in distant fields. Undoubtedly a chemist, concerned with the theoretical advances and the biological applications of his science, would have glanced at no less than 20,000 of those abstracts which in Chemical Abstracts were then increasing at a linear rate of about 1900 a year. For anyone of your special interests there would have been a background whose literature had been at least a century in the making and upon this background would have converged the theory, the technical appliances and the detailed information from various branches of knowledge. In the effort to appreciate these you would have sought among the 15,000 scientific texts published annually² such selected lists as those published by Nature and from over 3000 publications there recorded³ annually you might have glanced at the reviews of about 1000 in physics, chemistry, biology and bacteriology searching for texts and monographs which might help you to "keep up." You would have felt like Alice in Wonderland while the Red Queen cried, "Faster, faster. Here it takes all the running you can do to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!" You would have sought current reviews scattered in some three or five hundred journals which

² Allan Gomme in *The Uses of Libraries* (Baker editor), 1930.

³ *Nature*, 129, 370 (1931).

were to be found in an average medical library⁴ and which represented the cream of about 18,000 periodicals⁵ which were increasing at such a rate that one new journal of interest to chemists was created each fortnight⁶ and 150 new titles of publications in 16 different languages came within the field of "Botany: Current Literature" in the period 1920 to 1926.⁷

Of the despair of critical reviewers there is no lack of proof. One instance will suffice. In 1933 appeared a second volume of *Annual Review of Biochemistry*. The editor noted that the reviews covered a period of only one year and only 25 subjects and yet required the summary of 3000 papers. He estimated that these 3000 papers represented less than half of the papers which might have been of sufficient merit to have deserved treatment. Ten of the 25 authors were frank enough to state either that they had placed restrictions upon their already special topics or that they despaired of a complete analysis of the work for the preceding year.

Whence came the flood of scientific papers? The Industrial Revolution of the nineteenth century had created a demand for the scientific control and the investigation of industrial processes. It had also brought wealth and leisure, conditions conducive to the support of abstract investigations. Consequently old and new, academic and commercial institutions in the older industrial countries increased their production of scientific works enormously. In 1933 Gowland Hopkins⁸ estimated that 8 to 10 individuals were engaged in research where one was engaged twenty years before. But this was only the beginning. By the end of the first third of the 20th century scientific reports had begun to be abundant from the old cultural centers in China, Japan, India, and from educational, agricultural and industrial frontiers on every continent and great island of the earth. When a severe economic crisis made little impression upon the acceleration of the

⁴ Cunningham, *Science*, **77**, 410.

⁵ Allan Gomme, l. c.

⁶ Crane, *Ind. Eng. Chem. News Ed.*, **8**, 5 (1930).

⁷ Atwood, *Science*, **65**, 255 (1927).

⁸ *Science*, 1933, p. 229.

output and the circumstances of this crisis made it evident that ultimately there would be still more leisure for cultural pursuits throughout the World, it became evident that scientific literature at the close of the first third of the 20th century was growing like an infant in its second week after birth and that only the centuries which are to be its years of youth can witness all its changes on the way toward maturity.

Even now, near the close of the twentieth century, we cannot foresee the nature of a mature scientific literature, but it is well that we should examine the practices of the earlier period to appreciate the origins of the Scientific Reformation and problems that it left unsolved.

Previous to the world war it had been the custom to introduce a subject by reviewing its literature, preferably to the time of Aristotle. Increasing familiarity with ancient literature made continued reference to the priority of Aristotle somewhat embarrassing; but this was settled after the manner of the old decisions of taxonomy. An apparent end to the introductory literature review came after the world war when, in conjunction with the increased production already mentioned, there was a temporary increase in the cost of printing. Both of these forces made the *individual* journal cultivate a new type of scientific paper which has been called "the glorified abstract." It became the prevailing style. A wag maintained that he had seen in one editorial office an automatic machine geared to chop each comprehensive article at each sign of a change in topic. At any rate "the glorified abstract," with its tense confinement to the immediate business at hand, seemed to have eliminated the literature review and therewith a traditional, a clumsy but withal a useful way of giving the reader perspective in his placement of the subject. However, this condensation of the world's literature was apparent only. In the original literature exposition became so cryptic as to require review of each new group of original contributions and in a short time review journals so expanded as to open to second-hand reviewers their opportunity to learn a subject's content by compiling annotations of extensive bibliographies. Space that the

"glorified abstract" saved one journal was recaptured by the review journal.

The unsatisfactory natures of both the short, cryptic article and the second-hand review soon would have elevated to prominence that more mature style of writing which thoughtful authors were developing had there not been powerful forces opposing change. These we must examine.

It is obvious that, as the number of investigators in a field increases, the individual's chance of establishing a priority decreases. The defense left to the individual is increased frequency of publication. As late as 1933 there was a strong traditional sanction for this. It had its elements of genuine helpfulness and of lively interest. It protected, not against invasion of a reserved field, but the peace of the competent investigator who wished only notice that his work had been started long ago and was not to be confused with that of those who rushed to press. The worthy and the selfish motives kept alive the description of each, little advancing step long after simultaneity of publication had become so common that the prior claim had lost much of its distinction. By 1933 certain journals already had become virtually only a means of announcing claims or virtually only newspapers, and soon the volume of this newsy literature became so great as to make impracticable its inclusion in seriously critical reviews. This evolution to a neglect of the newsy literature inevitably led to a differentiation between professional scientists and a new sort of amateur. In the period 1935-40 there occurred a series of disputes centered about the claim that priority and "publication" are established by a radio broadcast *and* the circulation of its electrical transcription. The disputes were so lively that only newspapers could provide their adequate vehicle. Thus the daily press became the *traditional* vehicle. In 1938 a popular periodical called the *Daily Scientific Preview* made capital of this change in custom and by 1940 it was printing most of the more urgent, preliminary papers. But its greatest stroke came in 1942 when it opened a column and offered cash prizes for successful guesses upon the outcome of novel experiments. The fun of this new

game quenched hot disputes. A new and lively sort of popular science had been left in the wake of serious scientific advance.

A distinctive part of the literature was the contribution of students to whom publication meant an initiation into a privileged class. The important contributions from this source and their true functions in the scheme of educational and professional life were somewhat obscured by a practice best illustrated as follows. An investigator so noted for the number of his papers that experts in his own field had no time to read his work is reported to have made the following remark.⁹ "You complain," said he, "that I publish too many papers. But each paper represents an accepted means by which a student can acquire credit. That credit and his degree are certificates that the man is capable of earning his living in a highly organized system. I cannot deny my students their opportunity to earn their bread and butter and I am but following an accepted means of increasing the army of workers needed to exploit the resources of nature." The low standards thus inculcated were doubtless the cause of the remark that the army of graduates was like the army of Xerxes of which Thucydides said that "there were many men but few warriors." This was unfair alike to the university system at its best and even to its average product. But it is a commentary upon the times that certain universities let themselves be so impressed by the growing opinion that the doctorate had lost its significance that they did away with its bestowal. By the time a considerable number of their graduates were insisting upon the punctilious use of their title "Mr." other universities had already given enlarged meaning to the ancient traditions and their students were seeing to it that their theses would become creditable parts of their records.

Closely associated with the temporarily distorted attitude toward the function of publication in the training of graduate students was a strange opinion which developed in the rank and file of employees. This opinion was that an investigator's promotion depended upon the number of his publications. Professor Tobias

⁹ Confidential report.

Smith displayed discernment by the manner in which he investigated this matter. He first searched the minutes of faculty meetings. There he found unmistakable evidence that candidates did emphasize the number of their papers and no evidence that any committeeman ever read the *contents* of the papers. Thus it *seems* that the candidate was wise in his emphasis of number. But, suspecting that these records might not divulge all, Professor Smith searched the private correspondence of the time and discovered that members of the faculties, after requiring an indefinite minimum of publication, rated a candidate in *inverse* proportion to the number of his papers.

The prevailing attitudes had an obvious effect upon the literature of that period. Brief publication, sufficient to set forth the brilliancy of an idea, was at a premium. Indeed it appears to have been a period of exceptional brilliancy. But it should not be forgotten that the plodder and the master alike saw the cream of new developments skimmed and only the drudgery of careful, comprehensive work left to them. Out of this arose the well-known epithet "cream skimmer." It was a difficult psychological situation which was to be adjusted only as the purposes of a mature scientific literature developed. The development was by evolution in the course of which there were not lacking conscious efforts toward reform. These we must examine briefly.

Professor Goodview has recently examined the archives of several of the more reputable journals and has uncovered the vast, unselfish work of editors and their referees. The records show that editors agreed then as now that a scientific article should be treated as a potentially permanent stone in the edifice of a science. Avoiding evaluation of its final function, an editor must also regard an article as a potential object of scrutiny by innumerable readers so that a modest estimate of five or twenty thousand reader-hours for a paper of average importance would seem to call for at least a few hundred hours of careful composition. Yet there is some evidence, subsequent to 1935, that manuscripts were relayed to editorial offices by the teletypewriter directly after dictation. Professor Goodview finds that previous to 1935 there is abundant documentary evidence that editors and referees spared

no time in carefully compiling suggestions and corrections which were frequently ignored in authors' revisions. In return for their pains the editors received innumerable letters which they must have filed in grim silence since they are found filed without notes. Apparently to express his feelings one editor stored these letters in a disinfectant. Professor Goodview finds in the private correspondence of the editors evidence that they kept the faith, dreamed of clarity of exposition and even of literary beauty but resigned themselves to await the only effective remedy—the development of a general appreciation of what a mature scientific literature should be.

An attempt to solve the difficulty was made in 1946 by a committee of the League of Nations. It drafted resolutions entitled *Categories of Permissible Publications*. Unfortunately the subtitle, *Suggestions to Authors*, was printed in small type and was either overlooked or purposely ignored by the editors of commercialized scientific journals of the type that had started in Germany. Had the *Suggestions to Authors* been followed, practically none of these journals could have survived. Doubtless this is the reason for the propaganda against the League's *Suggestions* and the reason for their slow adoption. Containing nothing that could be called usurpation of individual freedom, the *Categories of Permissible Publications* set forth principles having universal appeal. For instance, the fourth category, subheading B, stated the principle that gratuitous speculation unsupported by experimental evidence or theoretical basis is unethical. Only gradually did this principle of ethics acquire force but it finally did so by way of the more conservative scientific societies until now it has such "teeth" that its violation is sufficient basis for exclusion from membership.

As the League of Nations' promulgation of the *Categories of Permissible Publications* failed of immediate effect so did the theoretical treatise by McKensie. McKensie shows that in the systems we study, the possible phenomenal relations among the components are practically infinite in number. It follows that if these are described according to even *one* code the possible record of so-called facts is practically infinite. The problem of scientific

publication, said McKensie, is not to provide *unlimited* facilities for the recording of "facts" important as they are. The very complex problem of publication must be governed by the two-fold principle that while the potential number of facts is infinite the intellectual abilities for their assimilation have inherent limitations. Even a cataloguing department of a science can ultimately assimilate only such facts as it can use in a practically available system of record and if the records become too elaborate their detail becomes less available than that supplied directly by nature. In many instances instruments have evolved to make qualitative comparisons and quantitative measurements so easy and so available to a particular purpose as to obviate the necessity for those written records which are necessarily limited in scope. By means of specific cases McKensie shows the ultimate futility of publishing what he calls the incidental, qualitative test. This is the sort of test which an investigator finds invaluable in quickly obtaining "leads" and "hunches" for systematic work. Any large, published accumulation of these defeats the purpose of a scientific literature, since their bulk becomes too great for fresh review and the apparent trend of their indications leans on habitual opinion.

With regard to the discovery of really new categories of relations McKensie shows that they are so rare as to impose upon the announcer the duty of a most exhaustive effort to demonstrate absence of correlation with the facts of established categories.

McKenzie recognizes clearly that during the early study of a subject a good plan of work may be missed, an adequate working hypothesis is seldom available and all pertinent relations may not be within the possibility of appreciation. But it then becomes the more important to publish only accurate and comprehensive data adequate for the purposes of a systematic organization which should be devoid of the generalized treatment for which the time is obviously not ripe. When a subject becomes stabilized the highly specialized ideas and confused intricacies which it has accumulated can and should be revised, and its generalization reduced to the simplest, most universal, most rigid and most basic terms possible. As Whitehead remarked "The paradox is now

fully established that the utmost abstractions are the true weapons with which to control our thought of concrete fact." This by no means implies stagnation or dogma for even the ordinary intellect now appreciates that generalizations are conveniences and that if there remains in any generalization lack of rigour or adequacy these are best revealed by scrutiny of the basic concepts. In the words of J. J. Thomson "a theory is a policy rather than a creed."

But no generation ever follows the thought of its own philosophers. McKensie's theoretical treatment, like the formal resolutions in *Permissible Publications*, was without immediate effect. Likewise without effect were the occasional examples of excellent scientific writing. An effective example is a reflector which focuses the light from an excited state upon its cause. Such an example came in 1965. Its true origin lay in that evolving change of attitude which I have reviewed; but its immediate incitement was a comparatively trivial incident which arose as follows.

As science became more highly specialized the coinage of new words became so rapid that in 1933 the rate was over one new word a day in medicine alone.¹⁰ At first this caused no worry. It was the general opinion that only the more useful words would survive to enrich the language of science. Then too, as Simeon Strunsky said, "Man has always had a capacity for adding to his happiness or to his terrors by substituting long words for short ones." As specialization became more refined there were more refined uses of the principle admirably expressed by Lavoisier: "Every physical science is formed, necessarily, of three things; the series of facts which constitute the science, the ideas which recall and the words which express these facts. The word ought to call forth the idea, the idea depict the fact; they are three impressions of the same seal." Now an elaborate terminology may prove invaluable to the specialist and so long as he is profitably occupied it would seem presumptuous to demand of him the abandonment of any convenience which serves his purpose. But, as I have already noted, the early part of the 20th century saw the property

¹⁰ Stedman, *Practical Medical Dictionary*, 12th ed.

of the specialist no longer exclusively his own. There was hardly a special development in any science which failed to find application in the practical affairs of the world. There was hardly a special development in an exact science which failed of theoretical use in such comprehensive subjects as biology or medicine. Failing to recognize this, specialists had made little or no effort to integrate their terminologies with those of contiguous subjects. As Stuart Mudd remarked, "The fact that the phenomena of bacteriology and immunity have been described in a special terminology has been a serious obstacle to the much needed collaboration of chemists." It was as if Jahveh had repeated his ancient words: "Behold the people is one and they have all one language—and now nothing will be restrained from them, which they have imagined to do. Go to, let us go down and there confound their language, that they may not understand one another's speech." As early as 1933 a biologist passing from a meeting of biochemists to a meeting of cytologists might have heard discussions of the same subject in languages very different.

By the nineteen forties the situation had become of some importance in the higher training of students. Perhaps it was trivial that students wasted time in learning words that contributed nothing to precision of thought or that lecturers wasted time in labeling each idea as it was developed. There will always be wastes of this or similar kinds arising from the conditions that make for progress. But what was serious was the fact that youths who came to learn subjects complained that they listened to logodaedalists. Let no one trifle with the idealism of youth or treat lightly those manifestations of its reaction to offense which he may think strange. The reaction to offense became apparent in 1947 when a class in immunology became riotous. In 1948 a large group of students at a prominent medical school conspired to write their examination papers in *Basic English*. They were failed. The furor which this caused in a sympathetic public precipitated the numerous student rebellions of 1949. The Federal Dictator, misunderstanding the situation, and believing that the cause was inadequate facilities for students to learn the languages of the sciences, ordered each national society to prepare

a dictionary of its terms. The Government was to pay for clerical work and publication but the society was to pay for intellectual direction. There was precedent for this division of cost. Industry had need for numerous compilations such as those of the older "Beilstein" and *International Critical Tables*, and, since these had grown so large as to exhaust the financial resources of contributing philanthropists, the Government had had to take over their publication. However, the Government had continued to use the volunteered services of "coöperating experts."

In outlining the project of a dictionary of bacteriology the committee of this Society discovered that no adequate dictionary could be written without the coöperation of experts from each exact and contiguous science. It also estimated that immediate publication would be impracticable since there was required of the lexicographer the formal definition of some special word for each, slight distinction. Not only would long, historical researches be necessary to find distinction between such terms as *microaerophilic aerophobe* and *prosaerotactic microaerophobe* but the utmost care would be required in defining little words such as *rough* and *smooth*. A legal outlet was found in the writing of a set of elementary texts the simplified language of which was declared the official language of the Society. The committee used this dodge in no mean spirit but only as the immediate and admittedly inadequate solution of a more fundamental difficulty which it had learned. For the solution of this difficulty it asked and received the support of this Society in a long-time, serious project, initiated by the committee's labor of 1952-1965.

Without a knowledge of the situation which I have attempted to outline it is impossible to appreciate the overwhelming task of that famous committee which began the work of the Scientific Reformation.

With splendid logic it attempted, first, to describe in simple language the basic relations in several branches of our science. But what were then considered the criteria by which a relation could be judged to be basic? The committee did well, first to indicate the difficulty in this question, and then frankly to dodge the answer. It chose to select, in the light of advanced informa-

tion, those relations which seemed at the time to be generally accepted as stabilized parts of knowledge. Type cases were presented as delineations of the subject. There was great difficulty in eliminating from many of these cases the very special hypothetical treatments in which they had become embedded, but this separation was essential to the better reselection which has been going on since.

Somewhat easier was the preparation of treatises upon the microscope, balance, potentiometer and numerous other instruments which were in common use and for which theory and practice had become fairly well stabilized. But here there remained the difficult task of tracing all concepts and mathematical formulas to their basic origins and building from these a well ordered theoretical treatment which raced smoothly to its conclusions. There were also many curious omissions to be made good. For instance, I myself have examined at random texts available in 1933 and have found: innumerable formulas for culture and staining media in which the specifications of materials were so indefinite that a chemist would not have been certain of the materials demanded; elaborate discussions of the mechanics of apparatus used in heat sterilization with no mention of the principles of heat penetration or of "thermal death rates;" descriptions of anaerobic methods with nothing concerning their physics or chemistry. Among ten elementary texts, two dictionaries, and two monographs dealing with the polariscope only one (an elementary text) defined the meaning of dextro rotation of the plane of plane polarized light and none mentioned the change in convention which had occurred in the nineteenth century! Among many texts which dealt with what was then called "pH" none gave its experimental origin and significance so clearly as to encourage the elimination of this convenient but unnecessary symbol and the adoption of the basic energy data which it crudely symbolized.

Perhaps the most difficult of all intellectual tasks is the construction of an elementary treatise on basic theory. Many of our ideas originate not in logical completeness but in response to repeated impacts of humble impressions. Theory then grows by

accretion and when completed it is often, as Eddington says, like *The House that Jack Buill*. Its basic definitions run in circles. To recast a body of theory, to reveal its basic postulates and its arbitrary beginnings, to construct its logical progression free from the entanglements occasioned by the accidents of history involves a heavy responsibility. If it is not met the teaching of advanced subjects suffers. Indeed, it is noteworthy that in a period when radical experiments in elementary education were numerous and when interest in the philosophy and methods of teaching was at its height, practically nothing had been done to so analyze the higher branches of learning that their greater and more *permeating* concepts would filter through to the elementary schools and there become the central themes of a consistent, systematic training. The reorganization of advanced thought was painfully slow for the simple reason that this task, which is essential to the organization of knowledge and to the development of a true pedagogy, is incomparably the most difficult of all intellectual tasks—a thousand times more difficult than the exposition of an advanced subject in terms which have become to the expert the parts of his daily speech.

An example which I give with temerity, because I am unfamiliar with its detail, will illustrate the sort of task which the committee undertook. There had been implicit in the early formulas for gravitational phenomena and in the early concepts of electrical phenomena a function which George Green in 1828 named *the potential function*. The concept of *the potential*, which in its origin at least is an extremely simple concept (that of a unit of potential energy) became invaluable in those branches of mathematics which were associated with physics. It was used throughout the higher physics in describing gravitational, electrostatic, electromagnetic and mechanical phenomena. In 1875 Willard Gibbs adapted the concept to the uses of chemistry; but accidents of history prevented it becoming well known among chemists. Consequently the biologist who took his views from texts of physical chemistry knew of *the potential* only as electrical potential. In his study of the distribution of water and of electrolytes in living things the biologist drew his ideas from very special

and not always appropriate laboratory devices, his ideas of chemical equilibria from an entirely different category of thought, his consideration of electrical potential differences at the phase boundaries of the living cell and at the phase boundaries of his electric cells from still other sources. Seldom was he guided to the view that his frequent measurement of an electrical potential illustrated the application of a generalized concept useful in each of these other subjects. Perhaps this is not a particularly fortunate illustration but it serves to indicate the sort of task the committee undertook. It searched for powerful, widely applicable and permeating principles which would serve in the coordination of theoretical subjects. It restored and adapted these to a well ordered elementary instruction which could progress smoothly into advanced subjects. It enlisted the services of the highest authorities and by irrefutable logic persuaded them to abandon many of the special conventions of their subjects which contributed little to precision of thought and nothing to general usefulness. No rigidity was sacrificed to mere simplicity, but simplicity was attained by clarity of thought and excellence of exposition. Perspective and proportion were preserved and each topic was integrated with those of contiguous subjects. Brevity and adequate completeness were joined in beauty of exposition. Not a principle was mentioned that was not carefully developed from its experimental origins or its basic postulates. Not a word was allowed that did not have its roots in the fundamentals of science. Not a book came to its binding before twelve years of coöperative and intensive labor.

The importance of these basic treatises lay not so much in their intrinsic worth, great as this was. The effort to write them was the first large scale, coöperative and pedagogically effective effort which had been made to organize the teaching of science, the first real recognition of an *impasse* facing teachers in the higher schools, the first recognition of an intellectual idealism on the part of students which had been ignored, scorned, offended by those who had forgotten the origins of universities in the will and idealism of students and who thought only of their own "papers."

The immediate enthusiasm for these basic treatises was slight as

it may well have been in an age which was only slowly recovering from an enthusiasm for the measure of greatness in terms of volume. But the enthusiasm was so sustained as soon to sweep through all departments. Then the effect upon the literature of science in general was remarkable. The series of basic treatises gave to the succeeding generation an almost universal appreciation of established type relations, of established theoretical principles and of permeating concepts of proven worth, so that authors could be confident that the basic origins of their own investigations would be appreciated and the language of their expositions understood. Theoretical investigations began to acquire a somewhat universal terminology and an ordered purpose. Gaps in knowledge which had been obscured by volume of publication, neglect of basic assumptions and the dodging of essential issues were more clearly seen and hence the more quickly filled. Dogmas were made transparent and the bodies of data which they had purported to describe were reorganized as bodies of data to await the perspicacity of genius. The vision of an integrated science makes trivial the newsy article, the preliminary paper, the incomplete investigation, the non-typical miscellaneous datum, the long table of unorganized results, careless reorganization of theory, unnecessary terms, the gratuitous speculative idea. The designer of instruments now leaves something to the common sense and ingenuity of the technician. There is now no occasion for an abstract such as the following, which was published in 1933. "The shield is made from a tin can."

A feeling for the foundations of science has merged with a new attitude toward the functions of publication. As the years pass, as the outline of the whole of science begins to take form, as the perspective of the past grows clearer and the hopes of the unlimited future grow brighter men find less satisfaction in the scrambles of the moment and a deeper joy in the ordered work of a lifetime. Few articles are now published which are not the work of long years—often the work of a lifetime, the results of comprehensive experiments thrice redistilled in redesign of method, in reorganization of data and in reflection. Scientific literature is on its way toward maturity.

I have given this brief historical review to remind you of the forces of evolution and of fitting example and to remind you of the prospect that science a hundred or a thousand years from now may suffer from our defects as we have suffered from the blindness of a preceding age.

The change of emphasis from factual detail to factual type, from theory suitable for "vanishing particulars" to theory as a permeating matrix has made us careless of the "conflicting fact" and neglectful of the genius who speaks an obscure language. Our assurance of an ordered future is too much like that which closed the nineteenth century. When the discoveries of the early twentieth century wrecked that old assurance men tried to "cut the wreck loose with an ax." They declared that Einstein completely replaced Newton, that Rutherford completely replaced Dalton, that Debye completely replaced Arrhenius, that DeVries completely replaced Darwin. Our renewed confidence in the irreversible achievements of science may be as smug as the attitude in 1933 which was to the effect that each advance upset all that had gone before. We shall doubtless have with us always those lesser ones who will expound only in terms of their own convenience and those others who will explain only by degrading the niceties of scientific thought to the style of a fairy tale. But this the Scientific Reformation has taught us—not to trifle with a literature that is to remain the record of emerging order. Henceforth we shall brook no compromise between simplicity and rigidity. Both qualities we shall carry to the highest ground of intellectual endeavor and there strive for a maturity of scientific literature commensurate with our vision of its beauty in the centuries to come.

LETTER FROM DR. THEOBALD SMITH

Dr. Theobald Smith, at a dinner in his honor in Philadelphia more than a year ago, was asked about certain attitudes which had guided his research. His replies were so illuminating that one of the guests, Dr. E. B. Krumbhaar, subsequently asked if he would not elaborate them for the inspiration and guidance of the students of the University of Pennsylvania. Dr. Smith recently wrote the following letter in his own handwriting to Dr. Krumbhaar.

[COPY]

THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
DEPARTMENT OF ANIMAL PATHOLOGY
PRINCETON, N. J.

October 11, 33.

DEAR DR. KRUMBHAAR:

As we grow old we come to the end of an individual era in which we have tried "to do our part." We begin to realize the important function of the past in shaping the future. We also feel the at times numbing and soporific effects of that past to be gotten rid of.

It is not uncommon for the younger generation to criticize or even disregard earlier work because it is not complete from the more recent standpoint. No research will answer all queries that the future may raise. It is wiser to praise the work for what it has accomplished and then to formulate the problems still to be solved. It is not profitable to enter into controversies especially with those working in another geographic area or continent unless the material on which their researches are based, has been examined.

To those who have the urge to do research and who are prepared to give up most things in life eagerly pursued by the man in the street, discovery should come as an adventure rather than as the result of a logical process of thought. Sharp, prolonged thinking is necessary that we may keep on the chosen road but it does not itself necessarily lead to discovery. The investigator must be ready and on the spot when the light comes from whatever direction.

There are many to compete with the young investigator. Oppor-

tunities for research have been increased a hundred fold in the past half century. More and more our colleagues fail to understand our work because of the high specialization of research problems. We must not be discouraged if the products of our labor are not read or even known to exist. The joy of research must be found in doing since every other harvest is uncertain and even the prizes do not always go to the discoveries to which we would assign them. Research has deserted the individual and entered the group. The individual worker finds the problem too large, not too difficult. He must learn to work with others.

In bacteriology and pathology research is slowly receding from the ultra-practical point of view of the early leaders. One group thought it possible to catch all bacteria in transit from one victim to another and to suppress disease in this way. Another group thought that a vaccine could be prepared for every disease. We have learned much since then and have become quite humble. Our researches no longer lead straight to Public Health regulations. They are more elusive and difficult to fit into any scheme for decreasing the incidence of disease. We must be content with the vision of future usefulness.

In general, a fact is worth more than theories in the long run. The theory stimulates but the fact builds. The former in due time is replaced by one better but the fact remains and becomes fertile. The fertility of a discovery is perhaps the surest measure of its survival value.

What is one man's meat is another's poison in research as in other vocations. Temperament goes far towards deciding our course. In the three different environments in which I have spent my active life I have always taken up the problems that lay spread out before me in the new environment, chiefly because of the easy accessibility of material without which research cannot go on; for in the early years material and resources were exceedingly scant and this meagerness determined the direction and scope of all research. My interest in a problem usually lagged when certain results could be clearly formulated or practically applied. To continue and analyze still further every link of the established chain either failed to hold my interest or was made difficult or impossible for causes lying outside the problem. As I look back it is precisely these links that have provided innumerable problems to others. Each link has grown into a chain and the end of successive chain making is not in sight.

Sincerely yours,

(Signed) THEOBALD SMITH.

SCIENTIFIC PROCEEDINGS
THIRTY-FIFTH ANNUAL MEETING OF THE
SOCIETY OF AMERICAN BACTERIOLOGISTS
PHILADELPHIA, PENNSYLVANIA, DECEMBER 27, 28 AND 29, 1933
Headquarters: Bellevue-Stratford Hotel

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ABSTRACTS*

Report of the Committee on Bacteriological Technic: Progress During 1933.

H. J. CONN, *Chairman*; VICTOR BURKE, IVAN C. HALL, J. A. KENNEDY, BARNETT COHEN AND ELIZABETH F. GENUNG, *Members*.

During this year the Committee's policy of keeping the Manual up to date by frequent revision of its leaflets has been continued. During 1933 a new index to the Manual has been issued and Leaflets I, II, and IX have been revised. These revisions have been printed during 1933 in the journal *Pure Culture Study of Bacteria*. This journal is sent to those who subscribe to keep their Manuals up to date. There are at present about 550 subscribers to the journal.

In 1932 the expenses of starting this new journal were so great that no profits from it were turned into the Society's treasury. This year, however, 10 per cent of the receipts from subscriptions have been turned over to the treasurer, together with the usual 25 per cent on all sales by the Committee. There has been a noticeable increase in the amount of business done by the Committee over that of 1932; thus the total number of Charts sold during the 12 months ending October 31 were 21,300 and of Manuals 311, while the total sales during 1932 were only 14,800 Charts and 249 Manuals. As the result of this increased business we are glad to be able to report that we have turned into the Society's treasury nearly \$100 more than in 1932. This contribution is assisting the Society in avoiding an increase in dues for 1934.

The work on biological stains through the Commission on Biological Stains is still continuing. Much of the investigation during 1933 has been in regard to basic fuchsin for use in the Endo medium. This work has resulted in a paper presented elsewhere on this program by H. J. Conn and Mary A. Darrow.

GENERAL BACTERIOLOGY

G1. The Physiological Youth of Bacteria as Evidenced by Cell Metabolism.

H. H. WALKER, C.-E. A. WINSLOW, EVELYN HUNTINGTON
AND M. GRACE MOONEY, Department of Public Health, Yale
School of Medicine; New Haven, Conn.

In a peptone or glucose-peptone culture of *Esch. coli* through which

* This number of the JOURNAL has been edited by the Chairman of the Program Committee. Authors of the abstracts in the Scientific Proceedings have not seen proof, due to restrictions of time imposed by the printing schedule.

air is continuously bubbled, the yield of CO_2 per cell per hour rises from 40 mgm. $\times 10^{-11}$ during the first hour to 120 mgm. during the second hour and then falls to 20 mgm. during the fifth hour. In a peptone medium through which nitrogen is bubbled lag is prolonged and the maximum yield is 70 mgm. $\times 10^{-11}$ per cell during the third hour. A glucose medium through which nitrogen is bubbled shows a wholly different type of metabolism, with a peak yield of 210 mgm. of CO_2 per cell during the fourth hour.

Ammonia yields show even more marked evidence of increased metabolism during the late lag and early logarithmic periods. "Physiological youth" which, as other observers have shown, is also evidenced by increased cell size, increased susceptibility to antiseptics, increased oxygen consumption and increased heat production. It seems possible that the apparent increase in metabolic activity per cell at this period may be largely (perhaps wholly) due to increased cell volume. Variation in cell volume cannot, however, account for the larger differences in yield between the lag and logarithmic periods as a whole and the subsequent phase of stable population.

Approximate computations suggest that one cubic micron of bacterial substance (*Esch. coli*), during the first 4 hours of the culture cycle, may consume 20 to 50 mgm. $\times 10^{-11}$ of oxygen per hour and may produce 20 to 70 mgm. $\times 10^{-11}$ of CO_2 and 3 to 7 mgm. $\times 10^{-11}$ of ammonia nitrogen, liberating in the process 30 to 90 calories of heat energy per hour.

G2. Rate of Growth and Viability in Bacterium coli. JAMES M. SHERMAN AND GEORGE M. CAMERON, Cornell University, Ithaca, N. Y.

It has been shown that the young cells of *Bacterium coli* from cultures growing slowly have greater hardiness when subjected to various deleterious factors than have the cells of the same organisms from cultures growing rapidly. This increased viability was found when the growth rate was reduced by growth at low temperature, growth in a dilute medium, or growth in a medium of increased osmotic pressure.

It is suggested that the rate of growth may not only be of importance in the viability of individuals of the same species, but, from the standpoint of natural selection, equally important to the species. From a review of the literature it appears that those species of bacteria and higher organisms which have been able to adapt themselves to life under conditions unfit for most organisms have relatively slow growth rates.

G3. *Studies on the Micrometabolism of Yeast Cells.* ERNEST A. PRIBRAM AND LOUIS KOTLER, Department of Bacteriology and Preventive Medicine, Loyola University, Chicago, Ill.

(I) *The problem:* Micrometabolism (Fr. Wright) is the metabolism of the smallest constituents of an organism. It can be studied by microchemical, physicochemical and ultramicroscopic methods. The metabolism of an organism is the result of all the microanabolic and microcatabolic processes of its constituents, which are colloids and crystalloids. In using yeast cells for our studies we have the advantage of relatively simple micrometabolic processes occurring in or on a living cell. The object of these examinations was (1) to determine the influence of different types of yeast cells on a solution containing microchemical quantities of the saccharides dextrose, galactose and lactose; (2) to determine the influence of microchemical quantities of KCl, CaCl₂ and FeCl₃ on the absorption of microchemical quantities of dextrose by yeast cells.

(II) *The experimental method used:* Types of yeast: *Saccharomyces cerevisiae* and *Saccharomyces lactis*. Quantity: 1000 cells per cubic millimeter, counted in hemacytometer. Quantity of saccharides: 2 mgm. per 1000 cu. mm. of the suspension of yeast cells in an isosmotic NaCl solution. Determination of dextrose, galactose, lactose respectively in the cell-free fluid after 15 minutes contact. Method: Epstein's modification of Benedict's picric acid method, using the Kuttner-Leitz Microcolorimeter. Galactose and lactose were calculated after the estimation of the total quantity of sugar in the fluid. The salts were used in 0.1 N solutions, 0.02 cc. of which were added to 1 cc. of the suspension, yielding 0.039 mgm. of potassium, 0.04 mgm. of calcium, 0.056 mgm. of iron per milligram of dextrose or 500 cu. mm. of the fluid.

(III) *The essential results:* *Saccharomyces cerevisiae* and *Saccharomyces lactis* grown on dextrose media absorb more dextrose than galactose or lactose. *Saccharomyces lactis* absorbs more galactose and lactose than *Saccharomyces cerevisiae*. The presence of CaCl₂ decreases, the presence of KCl and of FeCl₃ increases the absorption capacity of yeast cells, FeCl₃ less than KCl. The studies will be continued with other salts and with yeast cells of different ages, of different types and grown on different media.

G4. *Production of Peroxidase by Streptococci and Its Possible Significance.* MICHAEL A. FARRELL, Lehigh University, Bethlehem, Pa., and Yale University, New Haven, Conn.

Peroxidase was demonstrated in all of 22 strains of streptococci ex-

aminated. The group consists of both non-pathogens and pathogens, several of the latter being recent isolations. The organisms were grown in hormone broth, infusion broth, and on dextrose agar. The cells were centrifuged, washed 3 times in physiological saline solution, taken up in 5 to 10 cc. saline solution and aerated for 2 hours.

In the determination of peroxidase certain recently suggested methods were compared with the customary guaiac and benzidine tests. A reaction mixture consisting of 2 drops of the heavy bacterial suspension, 2 drops of 3 per cent hydrogen peroxide, and 2 drops of the color reagent buffered to a pH of 5.2, gave a positive peroxidase reaction with 4 different color reagents. These were 2-7-diaminofluorine-HCl, ortho-tolidine, benzidine and guaiac, given in the order of their sensitiveness. Two important factors which govern the appearance of a positive reaction are intensity of the reducing action of the particular organism and the cell concentration. When these factors are controlled a positive peroxidase reaction can be obtained within from one to 5 minutes.

Peroxidase in streptococci is thermostable, withstanding autoclaving at 120°C. for at least 15 minutes. However, the formation of a positive color reaction is inhibited by such respiratory poisons as potassium cyanide, hydrogen sulphide, iodo-acetic acid and brom-acetic acid.

The presence of peroxidase in these organisms, which have been reported heretofore as not possessing a catalytic system of the Warburg type, reopens the question of bacterial respiration of the streptococci.

G5. The Synthesis of Carotene by Bacteria. M. A. INGRAHAM AND C. A. BAUMANN, University of Wisconsin, Madison.

Carotene, the principal yellow pigment of carrots and the precursor of vitamin A in the animal body, is synthesized by many bacteria. Various staphylococci, flavobacteria, corynebacteria and mycobacteria were found to produce this pigment in appreciable amounts. The presence of carotene was demonstrated by chemical, spectroscopical and biological tests. It was not found in any of the anaerobes which were examined. A yield of 0.58 mgm. per gram of dried cells was obtained with one organism. This represents almost twice as much as is contained in carrots. Since carotene production has always been associated with the presence of chlorophyll, it is of interest to observe that although bacteria resemble animals in their lack of chlorophyll, they resemble plants in their ability to synthesize carotene.

The carotene content of a given organism varies with age and cultural conditions. A maximum value is obtained shortly before the available source of carbon has been exhausted. In *Myco. phlei*, caro-

tene production and degradation are paralleled rather closely by lipid storage and utilization. On sources of carbon such as glycerol, which result in a high yield of lipoids, there is a corresponding increase of carotene. By changing the source of carbon, it has been possible to vary the production of lipoids from 4 per cent to 56 per cent of the dry cell weight. The corresponding values for carotene were 0.00 and 0.35 mgm. per gram of dry cells. Light, temperature and iron salts affected pigmentation only as they modified growth. In media buffered above pH 8.3, however, carotene production was minimized.

The response of vitamin A deficient rats to the feeding of cells containing carotene, could be entirely accounted for by the amount of carotene administered. No biological or spectroscopical evidence was found for the presence of vitamin A as such in any of the bacterial extracts examined.

G6. The Influence of Some Environmental Factors Upon the Thermal Resistance of Bacterial Spores. HAROLD R. CURRAN, U. S. Department of Agriculture, Washington, D. C.

The heat resistance of bacterial spores is now known to be subject to wide variations, depending upon a number of physical, chemical and nutritional conditions. Existing incomplete knowledge upon this subject is quite inadequate to explain the enhanced thermal resistance shown by many spore cultures.

In this work the heat resistance of spores was studied in relation to a number of environmental conditions operative in the natural habitat of bacteria. The cultures used were *B. cereus* and 2 other aerobic spore-formers isolated from spoiled evaporated milk. The relative proportion of resistant spores was determined by exposure of spore-suspensions to heat at 95°C. for 10 minutes, and subsequent cultivation on agar plates. The thermal death time was determined by the method of Esty and Williams.

Prolonged continuous freezing reduced the proportion of heat resistant spores and decreased the thermal death time. Spores alternately frozen and thawed and spores held continuously in water showed no appreciable differences in heat resistance. Prolonged continuous drying usually yielded spores of enhanced thermal resistance while spores subjected to alternate wetting and drying in comparison with spores stored in the wet condition were usually intermediate with respect to their thermal resistance. Thermal resistance of the spores increased with the temperature at which they were held up to the optimum tem-

perature of the organism. Higher temperatures resulted in a reduced heat resistance.

With most of the organisms studied aging up to one year increased the heat resistance of the spores. The resistance did not change materially within the first 3 months. Prolonged exposure to high concentrations of metabolic growth-products has a weakening effect upon spores and decreases their thermal resistance. This is shown by the fact that spores obtained after long cultivation on agar and broth were much less resistant than spores recovered from these media after short cultivation and held the remaining period in a moist or dry condition.

The heat resistance of spores formed and held on artificial media was compared with those formed and held in a natural environment, such as soil, oats, water, etc. Soil yielded the most resistant spores but oats, dry-surface, and water spores were all considerably more resistant than those recovered from agar and broth.

These results indicate that environment is an important factor contributing to the development of resistance in bacterial spores.

G7. *The Rate at Which Spores of B. mycoides* Flügge, *Suspended in Peptone Solution, Become Stainable.* B. C. BRUNSTETTER AND C. A. MAGOON, U. S. Department of Agriculture, Washington, D. C.

The rate at which spores of various strains of *B. mycoides*, under conditions suitable for germination, become stainable, has been quantitatively studied. Washed spores were suspended in peptone solutions of known concentration; after various time intervals smears were made, stained with safranin (using the same technique as for vegetative cells) and the percentage of stained spores was determined.

The rate at which spores became stainable under these conditions usually was quite rapid. In the majority of strains studied, from 40 to 90 per cent of the spores had changed so as to become deeply stainable with safranin after 10 minutes' contact with 1 or 2 per cent peptone.

Preheating spores at 80°C. for 15 minutes did not alter the capacity for rapid change. By the time the spores had reached the stainable state, they had lost their characteristic resistance to heat. Using the Barcroft-Warburg micro-respirometer, oxygen uptake by spores suspended in peptone solution has been observed in as short a time as 15 minutes after mixing spores and peptone solution.

The effects of acidity, temperature and concentration of peptone on the rate at which a spore population becomes stainable, are described.

It should be noted that all the strains of *B. mycoides* studied had a low resistance to heat.

G8. *Some Observations on the Ability of a Mold, or its Metabolic Products, to Inhibit Bacterial Growth.* ROGER D. REID, Pennsylvania State College, State College.

A species of mold, closely resembling but not identical with *Penicillium notatum* (Westling), apparently was able to inhibit certain bacteria when grown in the same culture dish. The filtrate obtained by passing synthetic or infusion medium cultures of the mold through a Berkefeld filter was likewise inhibitory.

Investigations to determine the nature of the inhibitory substance and the conditions which affect its production showed: (1) It is not identical with, but is closely related to, the pigment which develops simultaneously. (2) It is relatively thermostable. (3) It volatilizes under certain conditions. (4) It contains certain enzymes, notably amylase and catalase. (5) An increase in pH appears at the same time as the inhibitory substance. (6) Alteration of the pH of the filtrate does not affect the activity of the inhibitory substance when once formed. (7) Surface tension of the broth is lowered by the growth of the mold at the same time that the inhibitory substance appears. (8) Cataphoretic readings did not indicate a change in charge upon the bacteria inhibited nor was the rate of movement affected. (9) The oxidation-reduction potential of the broth was not altered by the presence of the inhibitory material. (10) It can be adsorbed upon activated charcoal. (11) Dialysis, distillation at low temperatures and precipitation with salts did not separate the inhibitory substance from the filtrate. (12) Action of light and certain gases during the incubation period prevented the formation of the inhibitory substance.

G9. *The Bacteriostatic Effect of Indol and Skatol.* LESLIE A. SANDHOLZER AND RALPH P. TITSLER, School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.

The inhibiting effect of indol and skatol in 2 per cent peptone (Bacto) water has been studied on 23 species of Gram-negative, aerobic, non-sporogenous bacilli belonging to the colon-aerogenes and enteric disease groups.

In the case of indol, 179 strains were used. All of the cultures were inhibited by a 1:1500 dilution and all showed growth in a 1:5000 dilution. Sixty per cent of the cultures grew in a 1:2400 dilution. While some variation occurred between strains, the range within which any single species was inhibited was narrow. No general correlation between sensitivity to indol and indol production by the organisms was apparent. The least resistant species were *Salmonella gallinarum* and

Salmonella pullorum although individual strains of some other species showed greater sensitivity.

In the skatol study 69 cultures were employed. All of the strains were inhibited by a dilution of 1:3000 and all of them grew in 1:6500 dilution. Forty-four per cent of the strains grew in a dilution of 1:4500 and 87 per cent showed growth in a 1:5000 dilution. Sensitivity to skatol was parallel to that of indol but in every instance skatol exerted a greater inhibitory effect than indol. The ratio of the inhibiting dilution of indol to that of skatol was approximately 1:1.5.

G10. *The Effect of Certain Tri-Phenyl Methane Dyes on Staphylococcus aureus and Bacterium coli communior.* MARY V. REED AND ELIZABETH F. GENUNG, Smith College, Northampton, Mass.

Two problems were under consideration: (1) the adaptability of bacteria to disinfectants and (2) the comparative bacteriostatic values of different lots and brands of the same dye. The investigations were limited to the adaptation of *Staph. aureus* to crystal violet and of *Bact. coli communior* to brilliant green. Sixteen samples of dyes, including crystal violet, gentian violet, methyl violet, malachite green and brilliant green, were titrated. Both *Staph. aureus* and *Bact. coli* were used as test organisms in order to compare the effect of the different dyes on a Gram-positive and a Gram-negative organism.

The "extrinsic" method was employed, that is, the dye was added to the medium which was plain agar containing no NaCl. Dilutions were made in distilled water from a saturated alcoholic solution of the dye.

Staph. aureus did not readily adapt itself to inhibiting concentrations of crystal violet and was quite stable in the presence of all the dyes.

Bact. coli adapted itself to inhibiting concentrations of brilliant green and was induced to tolerate stronger concentrations. Inhibiting concentrations of the dyes produced temporary involution forms in *Bact. coli* cultures. The types of variants were specific for each dye. These involution forms disappeared as the alkalinity of the dye-agar medium increased. The colony formation and the fermentation and agglutination reactions were slightly affected.

The bacteriostatic value of each dye remained fairly constant for the different lots and for the two brands investigated.

G11. *The Effect of the Oxidation-Reduction Character of the Medium on Initiation of Yeast Growth.* J. J. REID AND I. L. BALDWIN, University of Wisconsin, Madison.

A study has been made of the problem of obtaining initiation of

yeast growth with small inocula in synthetic nutrient solutions. A medium suitable for initiation of growth is not necessarily as suitable from the standpoint of total growth. Inocula of *Saccharomyces cerevisiae* ranging from 100,000 to 10 cells per milliliter were used and sucrose from various sources was employed. Potentiometric studies were carried out in connection with the growth initiation determinations.

The results obtained indicate that the oxidation-reduction character of the medium exerts a decisive influence on the ability of *Saccharomyces cerevisiae* to initiate growth. In support of this assertion, the following is submitted:

(1) The potentials of the mineral salt sugar media were found to vary with different samples of commercial sugar. Media made with those sugars which gave lower potentials allowed growth initiation with smaller inocula than media with sugars which gave higher potential readings. (2) Reducing agents were used to lower the potential of the medium and, after suitable adjustment of the potential, growth was initiated with an inoculum of 10 cells per milliliter. The untreated medium required an inoculum of 10,000 cells per milliliter to insure growth initiation. (3) Caramelization of the sugar by prolonged autoclaving lowered the potential of the medium and made it more favorable for growth initiation. (4) Sterilization of the sucrose and salts in separate solution with subsequent mixing gave a medium with a higher initial potential and it required a larger inoculum for growth initiation than was the case with a medium sterilized after mixing all constituents. The potential of the former medium was found to fall for a number of hours following sterilization and this fall was accompanied by a slight improvement in the medium for growth initiation. The potential of the latter medium was found to rise following sterilization, reaching an equilibrium with the atmosphere in about 6 days. This rise was accompanied by a change in the medium which made it less suitable for growth initiation.

G12. Mercurochrome as a Bacteriological Stain. MARIE ECKHARDT
CONKLIN, Teachers College, Columbia University, N. Y.

In a study of the effect of certain chemicals in varying concentrations and for varying periods of time on bacterial spores, we tried to follow microscopically, by stained smears, the changing conditions within the spores. In making slides directly from chemically treated spores, a dye having fair color stability is necessary, as most stains are affected so much by acid and alkali that we gain no reliable indication of the com-

parative changes in cell permeability or internal chemical composition. A 5 per cent solution of mercurochrome (sodium dibrom-oxy-mercuri-fluorescein) serves this purpose admirably. Increase in spore color with increasing concentrations or exposures is demonstrable, until a specific point of concentration or time is reached, after which disintegration and lack of stain retention occurs.

While mercurochrome has doubtless been used as a bacteriological stain, the lack of scientific literature concerning it may warrant the following additional notes. As a general stain for vegetative cells of bacteria and yeasts, it shows greater internal differentiation than most common stains, especially if viewed under reduced light; granules, beaded structures, colorless sheaths, and darker staining cores may be seen in some forms by one minute staining with 5 per cent aqueous mercurochrome. This solution is easily prepared, forms no precipitate, and is very stable (the commercial 2 per cent solution gives fair results).

Mercurochrome makes a good combination with malachite green for staining spores, requiring no background stain, and no specific decolorizing step. The spores retain the green color and the vegetative cells stain red with no blending of the colors when stained as follows: (1) Make smears and pass through flame. (2) Flood with 5 per cent aqueous malachite green for 5 to 10 minutes over steam. (3) Wash in running water $\frac{1}{2}$ minute. (4) Counterstain with 5 per cent aqueous mercurochrome; wash in water.

G13. A Method for Distinguishing Living from Dead Cells of Gram-Positive Bacteria by Stained Preparations. WM. C. FRAZIER AND A. J. BOYER, U. S. Department of Agriculture, Washington, D. C.

A culture of *Streptococcus lactis* was grown for 10 days at 30°C., the curd filtered out by means of filter paper and the filtrate sterilized by passage through a Berkefeld filter. Treatment of cultures of bacteria or of dried and fixed smears on slides with this lactic filtrate at 60°C., for one-half hour, changed part of the cells from Gram-positive to Gram-negative. Bacterial counts made by cultural methods were compared with direct microscopic counts of Gram-positive cells on slides previously treated with the lactic filtrate. When groups of Gram-positive cells were considered as units in estimating numbers of bacteria per cubic centimeter, the count so obtained usually agreed fairly well with counts obtained by cultural methods. Comparative counts are given of cultures in milk, broth, or milk powder of *Propionibacterium shermanii*,

Lactobacillus bulgaricus, *Streptococcus thermophilus*, *Micrococcus luteus*, *casei* and *citreus* and *Bacillus megatherium* and *subtilis*. In all of these cultures the deaths were the result of age and not of heat or chemicals.

The addition of the lactic filtrate to cultures of lactic acid bacteria greatly stimulated growth.

G14. *Semisolid Media in Cultivation and Differentiation of Anaerobes.*

ROBB SPALDING SPRAY, School of Medicine, West Virginia University, Morgantown.

This report deals with the further development of semisolid media based on the principle established by Pringsheim, Lignières, Hitchens, and others. Formulas and methods are described with which the anaerobic spore-formers may be cultivated and identified, without recourse to special apparatus for anaerobic conditions of growth.

The principle consists of the addition of 0.25 per cent agar to fluid media of special composition for the cultivation of organisms and identification by characteristic reactions. A stock culture medium is presented, together with media for testing nitrate reduction and for H_2S and indol. A sugar-free base for fermentation studies is described. The use of whole milk with a strip of iron is discussed.

The further development and application of this principle is advocated in the interest of simplicity of cultivation and rapidity of identification of the sporulating anaerobes.

G15. *A New Medium for the Isolation of Intestinal Pathogens.* EINAR LEIFSON, The Johns Hopkins University, Baltimore, Md.

The ideal medium for the isolation of intestinal pathogens should inhibit the growth of all normal intestinal bacteria. None of the media in general use even approach this ideal. The writer has developed a medium which seems to be far superior to any of those in general use. This medium inhibits completely all Gram-positive bacteria. It allows the growth of the lactose fermenting bacilli (*Aerobacter*, *Citrobacter*, and *Escherichia*) to the extent of only 0.1 to 0.01 per cent, generally the latter figure. Suspensions of normal feces, streaked on the medium, generally show no growth. Colonies of colon bacilli which develop are large, red and easily distinguished from those of the pathogens. *Proteus* is inhibited somewhat and generally forms colonies which have dark brown centers. *Pseudomonas aeruginosa* grows well on the medium and develops greenish-grey colonies which usually are distinguishable from the colonies of the pathogens. Dysentery bacilli

grow well with the exception of some Shiga strains. Dysentery colonies are fairly large and colorless. Typhoid bacilli grow fairly well and develop colorless colonies which upon prolonged incubation may develop brown centers. *Salmonella paratyphi* grows well and develops colorless colonies. Paratyphoid bacilli which produce H_2S have colonies with dark brown centers. The medium has the following formula: Infusion agar (agar 1.5 per cent, peptone 1 per cent, NaCl 0.5 per cent); sodium desoxycholate 0.5 per cent, sodium citrate 3 per cent, ferric ammonium citrate 0.2 per cent, lead acetate 1/150,000, lactose 1 per cent, neutral red 1/50,000, and pH 7.2 to 7.4.

G16. The Endo Medium as a Trapping Agent and Indicator for Aldehyde.

LUBOW A. MARGOLENA, Commission on the Standardization of Biological Stains, AND P. ARNE HANSEN, New York Agricultural Experiment Station, Geneva.

Attention is called to the fact that Endo's medium acts like a trapping agent when *Bacterium coli* is grown therein. The characteristic color is caused by acetaldehyde which normally is absent in cultures of *Bacterium coli* but invariably is found when sulfite is added. The "restored" color is chemically different from the original fuchsin. This can be demonstrated by the fact that the former cannot be shaken out with ether when picric acid has been added previously, while the latter goes into the ether layer.

Details of this investigation are being published in *Stain Technology*, vol. 8, p. 131, 1933.

G17. Can the Endo Medium be Standardized? H. J. CONN AND MARY

A. DARROW, New York Agricultural Experiment Station, Geneva.

One of the most puzzling problems which has faced the Commission on Standardization of Biological Stains is the standardization of basic fuchsin for the Endo medium. Some samples have proved satisfactory in the standard formula while others have not, and there has been very little correlation of their behavior with the chemical nature of the dye.

At last the problem seems to be solved; and it has proved so simple that it seems strange the solution had not been thought of before. Apparently almost any sample of basic fuchsin now on the market in America can be used in the Endo medium, provided the amount of fuchsin in the medium is in the right ratio to the sodium sulfite used for decolorizing it. The difficulty in the situation comes from the fact

that the standard formula adopted by the A. P. H. A. (as well as several other Endo formulae) calls for 0.5 cc. of a 10 per cent alcoholic solution of fuchsin (which is actually saturated) per 100 cc. of the medium. The amount of dye thus introduced into the medium can vary very considerably according to the nature of the dye salt, the amount and nature of foreign material present and the care which is taken in getting as much of the dye as possible dissolved in the alcohol. If the sample at hand is very soluble and the alcoholic solution is actually saturated before withdrawing the 0.5 cc., the ratio of sulfite to dye will be only about 3:1, which is too low for good results. If, on the other hand, the sample is less soluble or the solution is not permitted to become fully saturated, the ratio of sulfite to dye may be high enough (perhaps 10:1) to show a satisfactory reaction.

It has also been found that too high a ratio of sulfite to fuchsin is equally unsatisfactory, because it prevents the restoration of color. This fault never occurs with the standard formula but it does with some others in the literature. To avoid these difficulties, it is suggested that the Endo medium be made up with the use of 1 cc. of a 1 per cent alcoholic solution per 100 cc. of medium. If this is decolorized with the amount of sulfite called for in the standard formula (0.125 gm.), the ratio of sulfite to fuchsin is 12.5:1, which seems to be about the best. This formula has proved satisfactory with a great variety of basic fuchsins, some of which have been rejected in the past by the Biological Stain Commission as unsatisfactory in the Endo medium.

G18. The Problem of Favorable Culture Media for the Isolation of Bacterium granulosis. IDA A. BENGTSON, National Institute of Health, Washington, D. C.

An effort was made to determine whether culture media more favorable for the primary isolation of *Bacterium granulosis* than that hitherto used could be devised. Since secondary growth of the organism occurs readily, even on ordinary culture media, the problem resolves itself into determining whether growth may be enhanced by variations in the composition of the culture media. It appears reasonable that media which are more favorable for secondary growth may also be more favorable for primary isolation.

For determining differences in growth, serial dilutions of the growth on agar slants were planted on the various media. Observations were made as to the time of the appearance of growth, the luxuriance of the growth and the size of colonies on the solid culture media.

Studies were made of the effect of variations in the composition of the base medium used in the Noguchi horse blood carbohydrate medium for plates, and of the addition of various inorganic salts, carbohydrates, amino acids and other organic compounds. Disaccharides were found more favorable for growth than monosaccharides and of the amino acids tested asparagin and tryptophane were found favorable. Better growth occurred in the *Leptospira* medium of Noguchi when horse serum was substituted for rabbit serum.

G19. A Corn-Liver Medium for the Detection and Dilution Counts of Various Anaerobes. L. S. McCLUNG AND ELIZABETH MCCOY, University of Wisconsin, Madison.

Many specialized media have been proposed for the growth of anaerobic bacteria. The majority of these require for their preparation either materials which are difficult to prepare or to standardize, e.g. casein digest or tryptic digests of meat; or else natural material which must either be obtained fresh from a slaughter house or from animal dissection, e.g., brain, beef heart, and living tissue. The substitution of a simple medium which could be prepared from dry materials and yet one which would give satisfactory growth for a number of anaerobes would be of benefit to small laboratories and a convenience and economy to those doing routine examination for anaerobes. Such conditions are met in a medium made from ordinary whole corn meal, dried liver and water. One to 2 per cent liver (tissue from liver infusion medium dried at 55°C. and finely ground) and 5 per cent corn meal are well steamed, cooled, and tubed. The mash may be sterilized by autoclaving for 2½ hours, and, if care is used in bringing down the pressure slowly after autoclaving, short (6-inch) tubes may be used without blowing the plugs. This medium need not be freshly steamed previous to use, requires no special seal of any sort or incubation in an anaerobic jar, and satisfactory results will be obtained with about 5 cm. depth of medium.

The following anaerobes have been cultured in this medium with success: *Cl. botulinum* (Types A, B, and C), *Cl. oedematiens*, *Cl. sporogenes*, *Cl. chauvei*, *Cl. histolyticum*, *Cl. welchii*, *Cl. tetani*, *Cl. tertium*, *Cl. multif fermentans tenalbus*, *Cl. bifermentans*, *Cl. aerofoetidum*, *Cl. fallax*, *Cl. tyrosinogenes*, *Cl. putrefaciens*, *Cl. putrificum*, *Cl. tetanomorphum*, *Cl. centrosporogenes*, *Cl. sphenoides*, *Cl. pasteurianum*, *Cl. acetobutylicum*, *Cl. felsineum*, *Cl. multif fermentans* and various yet unclassified butyric organisms and anaerobic spore-bearing thermophilic bacteria from the

soil. A number of these cultures have also been tested in media previously recommended for anaerobes, including milk (with and without reduced iron), brain, beef heart, liver infusion, alkaline egg, tryptone egg, and corn mash (with and without reduced iron) and with a single exception (liver) the corn-liver medium is as good as or in most cases better than other media in support of growth as shown by the number of positive cultures from dilute inocula and by early appearance of growth. With certain cultures, dilution counts have been attempted with both spore and vegetative inocula and more consistent results have been obtained with the corn-liver medium than with others tested.

G20. Azochloramid, a New Selective Bactericidal Chlorine Compound.

FRANZ C. SCHMELKES, HENRY C. MARKS, ISABELLE B. ROMANS, ELIZABETH S. HORNING AND ALBERT F. GUITERAS, Research Laboratories, Wallace & Tiernan Products Inc., Belleville, N. J.

If a blank platinum electrode is immersed in solutions containing chlorine compounds, potentials can be measured which closely parallel the reactivity of chlorine compounds with organic matter. These measurements may be duplicated within reasonable limits of agreement. The lowest voltage observed in such solutions was found in a solution of Azochloramid (N-N-dichloro-azodicarbonamidine).

Absence of any reaction between Azochloramid and a series of amino acids (tryptophane, tyrosine, alanine, etc.) and only slight interaction between Azochloramid and cystine was in striking contrast to the rapid reaction between Dakin Solution (sodium hypochlorite) and these amino acids. Chloramine-T (Sodium paratoluenesulfonchloramid) occupied an intermediate position, being much more closely related to sodium hypochlorite in this respect than Azochloramid.

Azochloramid was found to be bactericidal in spite of the absence of reactivity with any but intensely reducing organic compounds. Such tests carried out according to a modified Hygienic Laboratory method, and numerous other procedures, showed that Azochloramid killed Gram-positive cocci (*Staphylococcus aureus*, *albus*; *Streptococcus hemolyticus*, *viridans*) as well as Gram-negative organisms (*Esch. coli*, *Eb. typhi*) and various fungi and yeasts (*Trych. interdigitalis*, *gypseum*, *violaceum*; *Microsporon audouini*, *Monilia albicans*, *Saccharomy. ellipsoides*).

It is suggested that the application of such a chlorine compound might provide a means of injuring cells containing a large amount of accessible

reducing matter in the presence of cells containing little accessible reducing matter.

In point of fact, solutions of Azochloramid in body fluids, such as milk, serum and pleural exudate, are surprisingly stable, particularly if compared with the generally reactive solutions of sodium hypochlorite and Chloramine-T. This difference tends to disappear in whole blood. It is thus possible to kill a wide variety of microorganisms in the presence of, and without injury to, certain living tissues.

In this investigation considerable importance was attached to the fact that in testing the bactericidal power of chlorine compounds, preferential acceptors for chlorine compounds (sodium sulfite) were added to the inoculum in order to arrest the action of chlorine. This prevented bacteriostasis in the culture medium. The failure to eliminate this phenomenon has been justifiably criticized in the testing of various germicides.

G21. The Germicidal Efficiency of Hypochlorite Solutions in the Presence of Chicken Manure. ERNEST C. McCULLOCH, Division of Health, City of St. Louis, Mo.

Hypochlorite solutions containing as little as 130 parts per million of available chlorine killed *Salmonella pullorum* at 8°C. in the presence of a 5 per cent suspension of chicken manure. The Reddish technic, modified by the employment of low temperatures and the addition of chicken manure, was used. Under parallel conditions a 3 per cent phenol solution was require to kill.

The coefficient of dilution and the temperature coefficient of the hypochlorite solution were low, and a definite lag before the exertion of the germicidal action was noted.

The data indicate that a new compound, probably a chloramine formed by the action of the hypochlorite upon the ammonium from the manure, was responsible for the germicidal action.

G22. The Survivor Curves Exhibited by Bacterial Spores in Chlorine Disinfection. DAVID B. CHARLTON AND MAX LEVINE, Iowa State College, Ames, Iowa.

Observations on disinfection of dried bacterial spores by chlorine compounds revealed survivor curves which were characteristic for the individual disinfectants studied. When employing hypochlorites there was a pronounced lag followed by a rapidly increasing death rate. With Chloramine-T and with chloramine (calcium hypochlorite to which

ammonium chloride was added), the death rates, though slightly increasing at the beginning of disinfection were almost constant. The difference in the form of the survivor curves suggests that the mechanisms of disinfection with hypochlorites and chloramines are probably fundamentally different.

G23. Acidophilus Milk at Room and Ice-Box Temperatures. LENORE M. KOPELOFF, JOHN L. ETHELLES AND NICHOLAS KOPELOFF, Department of Bacteriology, Psychiatric Institute and Hospital, New York, N. Y.

Is it more desirable from the standpoint of viability to store acidophilus milk in the ice-box or at room temperature? In an attempt to answer this question samples of acidophilus milk, prepared with a rough and a smooth strain of *Lactobacillus acidophilus*, and with initial acidities of 0.6 and 1.0 per cent, were stored at 4° and 20°C. Counts of 5 plates from each of 2 dilutions from duplicate flasks were made every 24 hours for 4 days and at intervals thereafter. The experimental error on 5 plate counts from a single dilution averaged 4.37 per cent.

The viability of a rough strain of *L. acidophilus* was found to differ from that of a smooth strain of *L. acidophilus* in milk cultures of the same titratable acidity at 4° as well as at 20°C. This finding is of major importance since commercial acidophilus milks are usually made with a mixture of rough and smooth strains.

The rough strain of *L. acidophilus*, which is the only one of proven therapeutic value, loses its viability more rapidly at 4° than at 20°C. With an initial titratable acidity of either 0.6 or 1.0 per cent the original count is diminished approximately 90 per cent in 3 days in the ice-box and between 20 and 75 per cent at room temperature.

The smooth strain of *L. acidophilus* loses its viability less rapidly than the rough strain but storage in the ice-box is more detrimental than at room temperature.

Commercial acidophilus milk may be stored at any temperature providing cognizance is taken of the diminution in viability of the given strain at the given temperature. The final count in the hands of the consumer should not be less than 100 million viable organisms per cubic centimeter when the dosage is 1000 cc. per day for therapeutic purposes.

Because of practical difficulties encountered in ordinary manufacture it is recommended that acidophilus milk be kept in a cool place, but not in the ice-box.

G24. *Lactobacillus bifidus* Tissier and its Biological Position in the Group of Aciduric Organisms. JAMES E. WEISS AND LEO F. RETTGER, Yale University, New Haven, Conn.

Lactobacillus bifidus was isolated with little difficulty from the feces of nurselings. It often comprised from 90 to 95 per cent of the fecal flora of infants which had been breast-fed from three to four days. An acid broth isolation method was developed and used with good results. When isolated directly from feces and grown in Veillon tubes containing glucose infusion agar, both *L. bifidus* and *L. acidophilus* exhibited a varied morphology, including branching; however, under standard conditions of artificial culture the morphology of these two organisms was quite definite and regular.

Contrary to many reports, *L. bifidus* proved not to be a strict anaerobe but, like *L. acidophilus*, a facultative anaerobe.

The cultural and biochemical reactions of the two lactobacilli were studied. Slight differences were observed in their colonial forms. Fermentation and agglutination tests showed so much variability between strains of the same type that they were useless in differentiating between the two types.

The two types of lactobacilli showed no marked differences in their deportment toward indol, phenol, lysozyme, bacteriophage and varied hydrogen-ion concentration.

L. bifidus differs slightly from *L. acidophilus* in being more viable on the ordinary media, in being a stronger fermentative organism, in being slightly more resistant to certain inhibitory agents, and in producing smoother surface colonies. Whatever differences do exist between *L. bifidus* and *L. acidophilus* are quantitative rather than qualitative. It is, therefore, suggested that *L. bifidus* be considered as a variant in a species of which *L. acidophilus* is the central type.

G25. A Taxonomic Study of "*Cl. putrificum*" and Its Establishment as a Definite Entity—*Cl. lentoputrescens*, Nov. Spec. STANLEY E. HARTSELL AND LEO F. RETTGER, Yale University, New Haven, Conn.

Since Bienstock first reported his *Bacillus putrificus coli* there has been some question as to the identity of his organism, although for a number of years it was recognized quite generally as a definite entity. Its position in anaerobic classification was regarded as uncertain by British bacteriologists during the World War, and subsequently by an increasing number of investigators. Reddish and Rettger applied the name

Clostridium putrificum to an anaerobe which had been isolated by Sturges and Rettger, and which gradually assumed a distinctive position among the anaerobes.

Cunningham recently revived the question as to the identity of *B. putrificus* Bienstock and *Cl. putrificum* Reddish and Rettger. He suggested the possibility of the latter being nothing more than *Bacillus cochlearius* of McIntosh and Fildes, although he was aware that these two avowed species differ in their morphology and their ability to attack native protein. Cunningham isolated and characterized an anaerobic organism which he believed to have more in common with *B. putrificus* Bienstock than does *Cl. putrificum* or any other organism heretofore described.

In the present work the authors undertook to investigate the cultural, morphological and biochemical characteristics of these disputed organisms, with a view to establishing their identity. After a searching review of the literature on *Bacillus putrificus* Bienstock, and because of inability to obtain trustworthy strains, the authors concluded that Bienstock's organism should be excluded from the comparative study, in view of the lack of a satisfactory basis for comparison.

An expedient method of isolation of *Cl. putrificum* was devised and various strains of this organism, so isolated, as well as several that were obtained from different laboratories, were used in this investigation. Neither *B. putrificus* Cunningham nor *Cl. cochlearum* digested egg meat medium, while *Cl. putrificum* Reddish and Rettger did; *Bacillus putrificus* Cunningham peptonized litmus milk and liquefied gelatin, while *Cl. cochlearum* did not attack either the casein or the gelatin; *Cl. putrificum* developed round terminal spores, while *Cl. cochlearum* and *B. putrificus* Cunningham had oval terminal spores.

That *Cl. putrificum* of Reddish and Rettger differs very materially from either *Cl. cochlearum*, or *B. putrificus* of Cunningham, became very apparent. Since it cannot be related definitely to any other species of anaerobe (including *B. putrificus* Bienstock) it seems highly desirable to rename *Cl. putrificum*. The term *Cl. lentoputrescens* is suggested. This term indicates one of the most outstanding properties of this organism, namely its slow (*lentus*) decomposition (from *putresco*) of native proteins. It appears to the authors that the use of a new name for this species will obviate much of the confusion that has existed regarding the relationship of this organism to other anaerobic bacteria.

G26. *The Classification of Acid-Fast Bacteria.* JANET R. McCARTER
AND E. G. HASTINGS, University of Wisconsin, Madison.

The growth from single cells of acid-fast bacteria in a liquid synthetic medium has been followed microscopically, using a technic similar to that of Kahn. The species studied were an unnamed acid-fast saprophyte and the avian tubercle bacillus. The former organism was well adapted to such observation as it grows almost uniformly in one plane only. Although the growth looks like that of a branching mold mycelium under a magnification of 440 \times , it is easily demonstrated with a magnification of 1475 \times that there is no true branching, and that reproduction occurs by binary fission. Pressure in a chain of organisms due to growth leads to the pushing of individual rods out of line, and thus the appearance of branching is given. Similar phenomena can also be noted with the avian tubercle bacillus, but the observations are more difficult to make since the organisms tend to lie very close to one another and to grow in more than one plane.

We have planted and watched about 100 single cells of the H37 strain of the human tubercle bacillus, but have not been able to duplicate Kahn's observations of a life cycle for this species.

A review of the literature has been made to find the basis for the placing of the acid-fast bacteria in the order *Actinomycetales* in Bergey's Manual of Determinative Bacteriology and in Lehmann, Neumann, and Breed's Determinative Bacteriology. This basis is found to be, apparently, in the observations of such early workers as Nocard and Roux, Metchnikoff, Coppen Jones, and Miehle, all of whom reported branching of tubercle bacilli. Their observations were based on stained preparations or on living mounts insufficiently magnified. It is suggested that the term *Mycobacterium* has little justification and that the acid-fast organisms should be classified as *Eubacteriales* until more conclusive evidence is furnished for their resemblance to fungi.

G27. *The Constancy of Essential Characters in Lactobacillus acidophilus.*

L. A. ROGERS, U. S. Department of Agriculture, Washington,
D. C.

A typical acidophilus culture grown through repeated generations on a variety of media showed some marked changes in morphology as pointed out by Smith and Wallgren (Jour. Lab. and Clin. Med., 18, 1932, 134), but the sugar fermentations and relation of growth to temperature remained constant. By selection of colonies it was possible in a few replatings to obtain from a typical rough culture a strain which gave

almost entirely smooth colonies. The smooth strain formed acid in milk much more slowly than the rough one, but multiplication was apparently more rapid. This was evidently due to the tendency of the rough strain to form chains and thus give an apparently low count.

G28. *Studies on the Escherichia-Aerobacter Intermediates.* RALPH P. TITSLER AND LESLIE A. SANDHOLZER, School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.

An extensive study of the physiological characteristics of 29 methyl red positive and Voges-Proskauer negative *Escherichia-Aerobacter* "intermediate" cultures has been made to determine their taxonomic relationships.

On the basis of citrate utilization, hydrogen sulphide production and the fermentation of cellobiose and alpha-methylglucoside, these cultures could be divided into 6 major groups: 9 cultures were citrate, cellobiose, a-methylglucoside and H_2S positive; 4 were citrate, cellobiose and a-methylglucoside positive but H_2S negative; 8 were citrate, cellobiose positive and H_2S positive but a-methylglucoside negative; 6 were cellobiose positive but citrate, a-methylglucoside and H_2S negative; 1 was citrate positive but cellobiose, a-methylglucoside and H_2S negative and 1 was H_2S positive but citrate, cellobiose and a-methylglucoside negative. However, the existence of 25 sub-groups was indicated by additional differences in motility, the production of indol or the fermentation of certain other carbohydrates. This would seem to indicate the heterogeneity of the group.

No single characteristic has been found which will differentiate all the members of this group from both *Escherichia* and *Aerobacter* cultures. The production of hydrogen sulphide will serve this purpose, however, with some cultures.

G29. *The Occurrence and Significance of So-Called Atypical Reactions in the Colon-Aerogenes Group.* LELAND W. PARR, The George Washington University, Washington, D. C.

Within recent years 4 genera of *Colon-Aerogenes* organisms have been constituted—*Escherichia*, *Citrobacter*, *Aerobacter* and *Klebsiella*. Evidence exists pointing to such close interrelationships between these organisms that emphasis should for a time be placed on building up of similarities rather than differences. This conclusion has been reached through a lengthy cultural study of *Colon-Aerogenes* organisms derived from occasional pathological conditions, from water and soil samples,

from "infected" pumps, and from normal human feces, examined fresh and after storage.

Escherichia coli may possess a well-marked capsule when isolated from sources involving unusual adaptations, but recently we have shown that this microbe may occur, persistently encapsulated, in a healthy human adult. This finding and the recent work of Edwards, of Grant and Rettger, and of the Smith group at Princeton, emphasize again the close relationship of *Klebsiella* to *Escherichia* and *Aerobacter*. Further, the finding of such mucoid coli in normal human feces emphasizes the futility of attempting to demonstrate by plate culture colony differences between *Escherichia* and *Aerobacter* sufficiently valid to warrant application in water analysis.

Escherichia coli, indol negative, or lactose deficient, are not uncommon in fresh normal feces and are not to be considered solely as evidence of remote pollution. They can usually be "trained" back to normal. This procedure should be applied to Gram-negative bacilli, obtained from pathological conditions presenting difficulties of diagnosis.

Citrobacter and *Aerogenes* have been encountered in human feces in such numbers as to warrant questioning the assumption that these organisms are non-fecal until decisive tests can be validated distinguishing within these genera between their fecal and non-fecal members.

Citrate medium has been found very reliable in work with Colon-Aerogenes organisms and methyl-red has been found satisfactory in dealing with *Escherichia* and *Aerobacter* but less constant for *Citrobacter*,—organisms usually methyl-red positive. An explanation of this methyl-red variation may lie in the fact that the metabolism of this group at times exhibits the property of "bleaching" methyl-red. The nature of this "bleaching" action is a subject for further study.

G30. *Dissociation in Yeasts.* F. W. FABIAN AND N. B. McCULLOUGH,
Michigan State College, East Lansing.

In an attempt to revive a desiccated agar slant culture of *Saccharomyces cerevisiae* Hansen, Saaz strain, a culture of diplococcus was obtained. Upon serial transfer in malt extract broth, this diplococcus form was gradually changed back into the original yeast form.

Single cell isolations of the following yeasts were made: *Saccharomyces cerevisiae* Hansen, Froberg strain, *Saccharomyces ellipsoideus* Hansen, *Willia anomala* Saito, and *Zygosaccharomyces mandshuricus* Saito. Cultures from these single cell isolations were then aged and serially transferred in LiCl broth; in brilliant green broth and in malt extract broth

containing different percentages of ethyl alcohol. They were likewise subjected to desiccation on gypsum blocks and incubated at various temperatures in malt extract broth.

A study of the influence of the various chemical and physical agents showed that when the yeasts were subjected to these agents certain definite forms appeared constantly. These have been designated as the smooth or S form, the rough or R form, the gonidial or G form and the transitional or T form. The salient characteristics of the various forms are briefly as follows: The S form is the normal form described in the literature for most species and possesses the morphological, physiological and cultural characteristics usually ascribed to the species. The R form of the yeast consists of greatly elongated cells which form dull rugose (wrinkled) colonies having a filamentous edge under low power magnification. The physiological properties of the R form are the same as those of the S form. The G form consists of cells greatly reduced in size from that of the S and R forms. They are asporogenic, differ somewhat from the S and R forms in the sugars fermented and produce an acid instead of an alcoholic fermentation. All the G forms except one liquefy gelatin. Upon initial isolation, they grow slowly producing colonies microscopic in size at the end of a week's incubation. When they become adapted to their environment, they produce a thin spreading colony. The T form, which is the transitional form between the S or R forms and the G form, consists of a highly refractile cell which produces the G form by the formation of a large number of minute buds on the periphery of the cell. The T form has never been cultured but has been observed repeatedly in hanging drop preparations.

After the R and G forms had been obtained and single cell isolations made from them, it was possible to revert them to the original S form by rapid transfer in malt extract broth. The R and G forms are fairly stable since cultures of these forms have remained in their original form for approximately a year with little tendency to revert to the original S form.

In some cases certain variants were obtained such as pink S forms which became mucoid in character certain black R forms and one G form which produced a bright orange colored colony.

Limited immunological studies indicate a closer relationship between the R and G forms than between the S form and these two forms.

The production of these various forms clarifies some points in connection with yeasts which have remained obscure such as scum production, certain defects in beer and unsatisfactory yields in the production of

industrial alcohol. It is likewise evident that alcoholic production which is associated with true yeasts is absent and is replaced by acid production in the G form of the true yeast. Likewise the G forms of all the yeasts studied liquified gelatin, except in one case, while the S and R forms did not.

G31. Coccus Forms of C. diphtheriae. THOMAS C. GRUBB AND STEWART A. KOSER, University of Chicago, Chicago, Ill.

A study of the coccus forms of *C. diphtheriae* indicated that these forms may appear spontaneously in cultures and can be produced at will with some strains upon certain types of media.

When *C. diphtheriae* was aged or transferred repeatedly in various media, an irregular alternation between bacillary and coccus forms was observed. It is believed that this may be more reasonably explained by intrinsic changes in the medium than by postulating a life cycle, since subsequent work indicated that a change of medium alone would produce similar morphological variations.

Coccus forms of the diphtheria bacillus were produced promptly by growing the organism in a liver infusion medium. These cocci reverted to bacilli when transferred to veal infusion agar, blood agar, Loeffler's medium, etc. Microscopic observations of the organisms on a hanging block of liver infusion agar in a warm stage showed that the cocci were produced by a contraction of the rod into a rounded, coccoid shape. Forty per cent of 57 diphtheria strains and 5 per cent of 39 diphtheroid strains showed this change on liver infusion agar. The virulence and toxigenesis of these coccus forms in liver infusion broth could not be determined satisfactorily since they readily reverted to the bacillary form. The coccus forms were not filterable through Berkefeld, N. W., or Seitz filters.

The methods employed by several other workers for producing coccus forms were successfully duplicated, and these forms were morphologically identical with those produced in liver infusion media.

G32. Dissociation and Filtration Studies with L. acidophilus. MARY E. RANEY AND NICHOLAS KOPELOFF, Department of Bacteriology, Psychiatric Institute and Hospital, New York, N. Y.

Since stable R variants have never been derived from S strains of *L. acidophilus* of intestinal origin, an attempt was made to force this dissociation by methods commonly employed in similar studies with other bacterial species. These included the action of bacteriophage on *L.*

acidophilus, serial transfers in lithium chloride broth and repeated marginal fishings from old colonies.

Variations of considerable interest in broth and agar transfers have also been closely observed in a recently isolated intestinal strain of *L. acidophilus*. Colonies have been examined by Kuhn's impression method.

Strains from minute colonies closely resembling those described as G by Hadley were repeatedly isolated from streaked plates of old broth cultures and were studied in filtration experiments together with ordinary S and intermediate strains. Seitz, Mandler and Berkefeld filters of various grades were employed. Serial plate transfers were made on all Berkefeld filtrates which were also incubated for 2 months in broth. Particular pains were taken to avoid the possibility of contamination and to further this end all the manipulations were carried out in a "sterile" transfer room. The media and technical procedures were controlled by appropriate cultural methods.

This investigation yielded negative results. It was impossible to force the dissociation of an intestinal S strain to a stable R variant by any of the methods employed.

The strains from minute colonies resembling Hadley's G type were not associated with a filtrable phase of *L. acidophilus*.

When growth from filtrates appeared it was proven by careful controls to be in the nature of a contamination.

G33. Dissociation of Cl. welchii. ALDEN F. ROE, The George Washington University School of Medicine, Washington, D. C.

An intensive study of colony types was made on one typical strain; 4 other strains were studied somewhat less intensively, and an investigation of certain characteristics of 60 additional strains of this species was made. The findings in only one strain are presented here, but they were quite uniformly confirmed by findings in other strains.

To realize uniform results in a surface colony study, certain phases of technique are emphasized as essential, such as producing exact anaerobic conditions in cultures and jars, drying the surface of agar plates prior to inoculation and controlling humidity in jars during incubation.

Many of the customary methods of inducing dissociation as described in the literature on aerobes were found to be unsatisfactory. For this species, plates made from aging colonies and certain broth cultures combined with selection of extremes were found to yield the best results.

Six colony types (including the normal S) were encountered. In an

effort to conform closely with the aerobe literature, they were termed: S (smooth, normal); SV (smooth, viscous); O (large, rough, unstable); SR (small, rough, filamentous, intermediate between S and R); R (small, rough, filamentous, non-aerogenic), and M (midget, resembling the G type). The different colony types were compared cytologically and culturally. Outstanding morphological differences were noted between the S type (single and diplo-rods), the SR and R, which were both markedly filamentous, and the M, which was a small granular or beaded cocco-bacillus.

A marked range in cultural reactions of the colony types was found in type of growth in broth, gas production and fermentation reactions. With regard to the latter, it was possible to demonstrate that variants derived from a subgroup I strain fell into subgroups II, III, and IV of Simonds' classification, thus presenting a partial explanation (thru dissociation) for such earlier findings.

Serological differences were noted between the S and R. All colony types excepting the M were found virulent for guinea pigs when injected subcutaneously or intraperitoneally. The filamentous types were observed to be slightly more virulent than the S type. Other comparisons are also presented.

The true relationship of the variants to the original S type was proven by their repeated derivation from it or each other, and by the ability of a single antitoxin to protect guinea pigs against infection. A bacteriophage could not be demonstrated.

Among 44 newly isolated strains, 75 per cent were found to be S, 18 per cent of the O variety and 7 per cent of the SR type, thus proving that variants occur in nature.

Evidence which would permit interpretation of the existence of a life cycle could not be found. The changes from one colony type to another were chiefly oscillatory rather than cyclic.

G34. The Correlation of Animal and Plant Bacterial Behavior and Imposed Culture Medium Environment. AGNES J. QUIRK, U. S. Department of Agriculture, Washington, D. C.

It will be shown that the behavior of animal and plant bacterial organisms and imposed medium environment appear to be correlated.

Bacterial behavior in this study implies the production of phenomena and pathogenicity induced under the imposed medium environment.

The phenomena induced are sometimes referred to as rough, smooth and intermediate types of bacterial organisms, bacteriophage plaque ap-

pearance, pigment production and variation, clearing and clouding of broth culture, precipitate production, etc.

The imposed medium environment consists of beef infusion media in the liquid and solid state with 3 widely separated pH values and of Thaxter potato agar.

Pathogenicity tests of the organisms growing on the imposed environment are limited to plant bacteria only.

The organisms subjected to culture on the imposed medium set-up are as follows: Plant—*Bacillus tumefaciens* (hop strain), *Bacillus phytophthorus*—Appel strain, animal—a bacterial parasite found on the corn borer (interesting as a pigment producer), *Staphylococcus pyogenes aureus* (human source), *Bacillus pyocyaneus* (human source), and bacterial organisms isolated from the heart blood of 50 different guinea pigs undergoing tests. (Work carried on by animal bacteriologists.)

G35. *Variation in Cl. tetani.* J. H. ORR AND G. B. REED, Queen's University, Kingston, Canada.

Cultures of *Cl. tetani* in hormone broth plated on hormone-blood-agar in Fildes jars may exhibit 3 types of growth: low convex colonies with entire margins and perfectly smooth surfaces, regarded as the S form, flat granular colonies with fimbriate to widely spreading rhizoid margins, which resemble the usually described colonies of *Cl. tetani*, regarded as the R form, and very flat wide spreading lace-like growths with delicate rhizoid margins. The 3 types show considerable stability on solid media but the 2 spreading types may be isolated from the S form by a prolonged series of colony selections. The antigenic toxigenic properties of the 3 types will be discussed.

G36. *Fungous Phases in Bacteria: Zygotes and Sporangia.* (With motion pictures.) JEAN BROADHURST, Teachers College, Columbia University, New York, N. Y.

Two fungus-like phases of bacteria are shown in hanging drop cultures: (1) A zygote phase in *Bacillus polygenes* suggesting the double zygotes of *Mucor*, in which two vegetative cells fuse, forming two globoid, refractile bodies after which the vegetative cells disappear, each globoid body developing into a compact, slowly-increasing mass of small globular cells. (2) A sporangium phase in *Serratia marcescens*, in which short and long stalked sporangia develop laterally on filamentous threads of *Serratia*. These sporangia are several times the diameter of the threads and contain a dozen or more globular bodies.

G37. The Artificial Production of a Specific Lytic Agent Which Behaves Like Bacteriophage. JOHN D. LEMAR AND JOHN T. MYERS, College of Medicine, University of Nebraska, Omaha.

Bronfenbrenner and others observed that increase of oxygen tension in the medium during bacteriophagy increased rate of lysis and formation of additional lysin. This suggests a possible relationship between oxidation and the formation of bacteriophage.

The following oxidizing agents were mixed with young broth cultures of *E. coli*, adding 10 per cent by volume of each agent, respectively: 10 per cent KMnO_4 , 10 per cent H_2CrO_4 , and 3 per cent H_2O_2 . No lytic filtrates were obtained.

When cultures of *E. coli* were autoclaved 20 minutes at 15 pounds pressure, and oxidizing agents added in the same concentrations, filtrates were not lytic. When a 24- to 48-hour period of autolysis occurred between autoclaving and addition of oxidizing agents, filtrates were obtained apparently capable of causing lysis of young, lightly seeded cultures. Oxidized sterile broth filtrates were also lytic due to the oxidizing agents. However unpublished work indicated that bacteriophage could be extracted in part, unaltered, by ether, which suggested a method of removing the oxidizing agents. When ether extracts of killed, autolyzed and oxidized cultures were evaporated over sterile broth and added to young cultures of the same organisms, there was lysis in the case of oxidation by peroxide but not with the other agents. Such extracts resembled bacteriophage as follows: serial transfers for 14 generations continued to produce lytic filtrates, the titer being increased. Exposure to 75°C . for 30 minutes destroyed lytic power. Lysis occurred in dilutions up to 10^{-10} . Filtrates withstood freezings and thawings without diminution of potency. They were specific. Ether extracts of oxidized sterile broth did not yield a lytic filtrate. Specific lytic agents were prepared in the same manner against a number of organisms.

Optimum results were obtained by autoclaving a 48-hour culture and permitting it to autolyse for 48 hours, followed by 48 hours oxidation with 15 cc. of 3 per cent H_2O_2 per 100 cc. of culture.

Oxidation of living cultures or autolysis of killed cultures without oxidation failed to produce a lytic agent.

G38. Streptococcus Bacteriophage and Its Usefulness for the Identification of Strains of Hemolytic Streptococci. ALICE C. EVANS, National Institute of Health, Washington, D. C.

Three races of streptococcus bacteriophage are described, one of which

was recently isolated by the writer. The 3 races differ in the size of the plaques formed on agar cultures; they differ slightly in thermolability; they are serologically distinct, and they differ in respect to the strains of streptococci which they can attack, only 5 out of 284 strains studied having been found sensitive to the filtrate of more than one of the phages. In the nascent state, that is, in the presence of a sensitive strain, each one of the 3 phages is capable of attacking a much greater number of strains than are sensitive to the lytic filtrates.

The reaction of a strain of streptococcus to the 3 phages in both the nascent and filtered states gives 6 characters which may be recorded in signs in a specified order. The pattern of signs for any given strain offers a characteristic for comparison with other strains. A correlation of the lytic patterns of the various strains of streptococci with the time and place of their isolation was found. This suggests that sensitivity to bacteriophage should be useful in the identification of strains, particularly during epidemics when rapidity of identification is important.

G39. The Adaptation of Staphylococcus Bacteriophage to an Artificially Produced Anti-Staphylococcus Bacteriophagic Serum. MORRIS L. RAKIETEN, School of Medicine, Yale University, New Haven, Conn.

An anti-staphylococcus bacteriophagic serum (rabbit), having a titer of 1:3200 was produced by intravenous inoculations of polyvalent staphylococcus bacteriophage NH10.

Using a method which consisted in adding bacteriophage and susceptible bacteria to increasing concentrations of anti-serum it was possible to adapt phage NH10 so that it brought about lysis of the susceptible strain in a concentration of 1:650 of the anti-bacteriophagic serum. However, this method was uncertain since one could not be sure that the adapted phage would bring about lysis in the same concentration of serum upon repeating the procedure.

A better method permitted us to adapt phage NH10 so that it brought about lysis of the susceptible strain in a concentration of 1:15 of the artificially produced anti-bacteriophagic serum. The procedure consisted in adding a small amount of phage to known dilutions of the anti-serum, incubating from 15 minutes to 3 hours at 37°C., removing 0.02 cc., adding it to a culture of the susceptible bacteria, mixing, and plating 0.02 cc. on a Savita agar plate. (Practically all of the neutralization of phage by anti-bacteriophagic serum takes place within 3

hours.) Plaques were picked from the segment showing the largest number of plaques in the highest concentration of anti-serum in the longest period of time. The number of plaques showed a real increase with each successive contact in the same concentration of anti-serum over increasingly long periods of time. This method permitted us to pick the bacteriophage corpuscles which showed their ability to withstand the neutralizing action of the anti-serum.

During the course of the adaptation it was observed that not all of the plaques were equally susceptible of being adapted to increasing concentrations of the anti-serum, that in concentrations of the anti-serum from 1:100 to 1:25 plaques suggesting agglutination of the bacteriophage corpuscles appeared, and that some of the plaques which resisted high concentrations of the anti-serum seemed to be composed of corpuscles which had lost their lytic quality.

The adapted bacteriophage is as heat stable as it was originally; it still retains a high degree of polyvalency; and it is able to bring about lysis of susceptible strains of staphylococci in concentrations of human sera that are definitely inhibitory to other unadapted staphylococcus bacteriophages.

AGRICULTURAL AND INDUSTRIAL BACTERIOLOGY

A1. An Extremely Economical Sugar Fermentation. H. J. CONN AND MARY A. DARROW, New York Agricultural Experiment Station, Geneva.

In the course of work on those soil bacteria which produce only minute colonies in either gelatin or agar it was realized long ago that acid production from sugar could be observed with these organisms only when growing them on a medium of relatively low buffer content. Even on such a medium it has been noticed that the same organism may sometimes show acid and other times not. These observations have led to a study as to what the actual products of fermentation by these bacteria may be.

A more careful study has now been made of one of these organisms, *Bacterium globiforme*, previously described by one of the writers. The amount of carbon dioxide given off during the fermentation has been determined, as well as the amount of sugar after growth of the organism. Carbon dioxide and sugar also were determined in an uninoculated check. At the same time the growth produced by the bacteria in this medium has been separated out by means of a centrifuge and has been

weighed. It was found that these bacteria use the sugar very sparingly and convert all but about 40 per cent of that used into carbon dioxide. The maximum amount of carbon dioxide production occurred with only about 0.1 to 0.2 per cent of glucose.

With 0.15 per cent glucose it was observed that the sugar consumed, which was not converted into carbon dioxide, would yield only about enough carbon for the amount of cell substance actually present in the growth produced by the bacteria. This leads to the conclusion that these soil bacteria are extremely economical in their use of sugar. They seem to be adapted to produce the largest amount of growth and to obtain the greatest amount of energy possible from a minimum quantity of sugar. This undoubtedly adapts them to soil conditions where sugar is never present in any quantity and can be obtained only as other organisms split the higher carbohydrates.

A corollary of this conclusion is that in the case of organisms like these (that produce no acid in sugar fermentation except carbon dioxide), the use of indicators to show whether or not fermentation takes place is a procedure which requires a great deal of caution. A rise of H-ion concentration may easily be masked by the production of alkalinity if some other compound like potassium nitrate is present from which the bacteria utilize the anions to a greater extent than the cations.

A2. Cultivation of Organisms Concerned in the Oxidation of Thiosulfate in Mineral Media. ROBERT L. STARKEY, New Jersey Agricultural Experiment Station, New Brunswick.

Representatives of the group of bacteria commonly referred to as "thiosulfate bacteria" were detected in all of 28 soils which were examined. From only one soil did growth develop which was characteristic of *Thiobacillus thiooxidans*. However, after once becoming established, this organism persists for long periods in soils containing elementary sulfur. Organisms which have been isolated from these soils are responsible for three distinct types of oxidation of thiosulfate. One (*Th. thioparus* Beij.) precipitates considerable sulfur, increases the acidity and develops rapidly. A second (A) increases the acidity without precipitation of sulfur. The third (B) brings about a decrease in acidity with no appreciable sulfur precipitation. Organism A produces a uniform turbidity in the solution while B shows no pronounced evidence of growth. Culture B is closely related to Trautwein's organism (*Th. trautweinii*, Bergey).

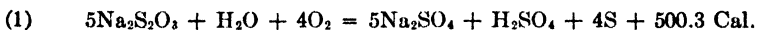
Culture A is favored by initial alkaline reactions; culture B develops

best at slightly acid reactions; *Th. thioparus* grows well in either slightly acid or alkaline media. Media strongly buffered with phosphates favor oxidation by A and B but have little favorable effect on *Th. thioparus*. Cultures A and B persist in solution media for long periods without transfer; *Th. thioparus* dies out if not transferred frequently. Organic substances favor oxidation of thiosulfate by B, but have little or no favorable influence on A or on *Th. thioparus*. Considerable concentrations of organic substances are tolerated by each organism. Both A and B develop on organic media in absence of thiosulfate. *Th. thioparus* does not develop in organic media free from thiosulfate. Cultures A and B do not lose their capacity to oxidize thiosulfate after continuous cultivation upon nutrient agar for 12 months.

Various fungi and actinomyces cause practically no changes in thiosulfate either in media containing organic substances or free from organic materials. Of 15 bacteria studied, three gave evidence of thiosulfate oxidation; *Ps. fluorescens*, *Ps. aeruginosa* and *Achrom. stutzeri* oxidize thiosulfate the same as culture B. It is concluded that culture B is not an autotrophic bacterium, and that transformation of thiosulfate is incidental to its normal nutrition and is of no vital importance as a source of energy in its metabolism.

A3. Products of the Oxidation of Thiosulfate by Bacteria in Mineral Media. ROBERT L. STARKEY, New Jersey Agricultural Experiment Station, New Brunswick.

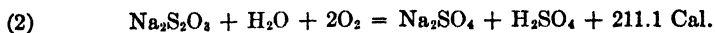
Analyses of culture media supporting the development of a sulfur bacterium which appears to be *Thiobacillus thioparus* Beij. indicate that thiosulfate is oxidized with the formation of sulfate and elementary sulfur. Sixty per cent of the thiosulfate sulfur appears as sulfate and 40 per cent as elementary sulfur, no other sulfur products being formed. The medium becomes acid as is apparent from the following reaction:



The organism assimilates carbon from HCO_3^- to the extent of 1 gram of carbon for each 125 grams of thiosulfate sulfur oxidized. About 4.8 per cent of the energy released is utilized for the synthesis of cell material. This organism appears to be a strict autotroph. It is a Gram-negative tiny rod, 0.3 to 0.7 by 0.4 to 0.9 μ in size, apparently non-motile.

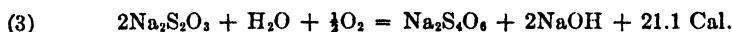
A second organism (A) oxidizes thiosulfate to sulfate, this being the

only sulfur product appearing. The hydrogen-ion concentration of the medium increases as growth proceeds.



Since a greater amount of energy is liberated per unit of thiosulfate oxidized, the ratio of thiosulfate sulfur oxidized to carbon assimilated (48/1) is smaller than for *Th. thioparus*. About 5.7 per cent of the available energy is utilized for cell synthesis. This organism is a facultative autotroph, developing on either mineral media containing thiosulfate or upon various organic media free from thiosulfate. It is a small, Gram-negative, non-motile rod, 0.4 to 0.8 by 0.6 to 1.8 μ in size.

Thiosulfate is less completely oxidized by a group of organisms, a member of which has been named *Th. trautweinii* Bergey.



Tetrathionate appears to be the initial sulfur product formed. This subsequently breaks down and various substances appear such as pentathionate, trithionate, sulfate and elementary sulfur. The hydrogen-ion concentration is quickly lowered in the initial stages and then becomes somewhat greater during secondary transformations. No carbon assimilation has been noted and it is believed that the organisms effecting the change are strictly heterotrophic. Very little energy is released by the reaction there being only 5.2 Cal. per gram atom of thiosulfate-sulfur as compared with 50.0 Cal. for reaction (1) and 105.6 Cal. for reaction (2). The organisms were Gram-negative motile rods; one organism was 0.3 to 0.7 by 0.4 to 1.2 μ and others were 0.4 to 0.9 by 1.0 to 3.0 μ in size.

A4. Strain Variation of *Azotobacter* and the Utilization of Carbon Compounds. NATHAN R. SMITH, U. S. Department of Agriculture, Washington, D. C.

By plating soil on a nitrogen-free carbohydrate medium strains of *Azotobacter chroococcum* were isolated which were not able to utilize mannitol as a source of energy. Since mannitol medium is usually used for the isolation of *Azotobacter*, it is evident that it may be selective and that non-mannitol fermenters may be over-looked. Variations in the ability to use dextrin, starch and glycerine were also found, regardless of the isolation medium. All strains used dextrose and sucrose. Two strains of *Azotobacter vinelandii* and one of *Azotobacter*

agilis obtained from various laboratories utilized dextrose, sucrose, mannitol and glycerine but could not use lactose, starch and dextrin. This lack of variation in their carbon requirements probably means that these cultures had a common origin. *Azotobacter beijerinckii* may be considered as a white mutant of *Azotobacter chroococcum* and *Azotobacter vitreum* a colorless variation of *Azotobacter vinelandii* on account of the utilization of carbon compounds as well as other characteristics.

A5. *The Nodule Organism of Mimosa pudica L.* LEWIS T. LEONARD,
U. S. Department of Agriculture, Washington, D. C.

The sensitive plant, *Mimosa pudica L.*, a novelty in the north and a pest in the tropics, has an association with organisms which differ in some respects from the common nodule bacteria. They are most readily isolated with slightly acid or neutral beef agar, tolerate reactions between the pH limits of 5.5 and 10.0, grow poorly on the common media used for nodule bacteria and are apparently not interchangeable with any of the common species of nodule organisms.

On peptone containing any other media, this organism produces an odor resembling that of trimethylamine.

Following the tendency to split the nodule bacteria into species, it is apparent that the organism of *Mimosa pudica L.* may well be given a new species name when its description is published.

A6. *Concerning the Fixation of Nitrogen by Germinating Seeds of Leguminous Plants.* F. S. ORCUTT, A. M. SHANNON AND P. W. WILSON, University of Wisconsin, Madison.

During the past year, Nerina Vita at the University of Bologna, Italy, has published experiments in which it is claimed that seedlings of peas, horse bean, and lupines subjected to certain treatments, can fix atmospheric nitrogen without the aid of bacteria. These treatments include exposure of the seeds during the germinating process to weak solutions of magnesium sulfate, potassium sulfate, manganese sulfate, caffeine or strychnine nitrate. On the 5 grams of seed used for each treatment the author reports gains as high as 50 mgm. of nitrogen or about 30 per cent increase in the total nitrogen present in the pea seed.

Attempts were made to duplicate these results. About 2.5 grams of peas were weighed into a sterile petri dish, sterilized, transferred aseptically to a sterile, cotton-plugged, Kjeldahl flask, and treated with 50 cc. of sterile solution of one of the following: magnesium sulfate, 0.0177 and 0.0737 gram per liter; manganese sulfate, 0.00246 and 0.0246 gram

per liter; caffein, 0.5, 0.75, 1.00, and 1.5 grams per liter; strychnine nitrate, 0.75, 1.00 and 1.5 grams per liter. Fifteen samples of each treatment were placed in the greenhouse under diffused light and allowed to germinate; a like number of controls treated with sterile distilled water were included. At the end of 7, 11, and 13 days, 5 samples of each treatment were removed for total nitrogen analysis.

It was found that the nitrogen content of pea seeds showed great variation, and therefore it is advisable to take at least 5 samples in an experiment of this nature in order to determine the standard error associated with the estimate of the nitrogen content of the seeds. When this was done no evidence was obtained of fixation of atmospheric nitrogen by peas exposed to the various treatments.

A7. The Persistence of Tubercle Bacilli in Soil and the Effect of Various Soil Microorganisms on Tubercle Bacilli. C. RHINES, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

The *Avian 531* strain of tubercle bacilli was inoculated into normal soil, into toluol-sterilized soil, and into soil sterilized by heat. Reisolation and determination of numbers of tubercle bacilli in the soil were attempted at various intervals, using the plate method. No reisolation was achieved from untreated soil, because contaminants overgrew the plates and because soil mycobacteria interfered with the identification of *Avian 531*. For a period of one month *Avian 531* maintained a constant population in toluol-sterilized soil. The numbers of *Avian 531* increased notably in soil, as determined by the plate count, when present in pure culture, and when associated with *Aerobacter aerogenes* or *Bacillus megatherium*. The fungus *Verticillium chlamydosporum* depressed the numbers of the tubercle organism. A pure culture of *Avian 531* was grown, together with a pure culture of *Colpoda steinii*, a soil ciliate. The ciliate, incapable of maintaining existence on any bacteriological medium tried, has persisted for more than a year in nutrient broth suspensions of *Avian 531*. The ciliate ingested the tubercle organism. No evidence, however, was procured to show that the ciliate actually digested the mycobacterium.

A8. A Serological Study of Certain Butyric Anaerobes of Soil. ELIZABETH MCCOY, University of Wisconsin, Madison.

Bredemann in 1909 classed the 27 cultures which he studied as one species, *B. amylobacter* A. M. et Bredemann, regardless of their previous

designations in the literature. The third edition of Bergey's Manual shows the continued tendency to group together all nitrogen-fixing soil butyrics; *Cl. butyricum* Prazmowski is there proposed as the type species of the genus *Clostridium* and probably synonymous with many of the supposed "species" in the literature. Certain of these historical cultures have been obtained from sources which are believed to be reliable. They have been carefully tested for purity and used for immunization of rabbits for agglutination studies. Sera for the following have been prepared: *Cl. pasteurianum* Winogradsky, *Gran. butylicum* Beijerinck, *B. butylicus* Fitz, F. B. Fernbach, *Cl. felsineum* Carbone and *Cl. acetobutylicum* Weizmann.

Direct and cross agglutination tests and absorption of specific agglutinins showed identity of *B. butylicus* and the Fernbach bacillus. In the same way, antigenic identity of *Gran. butylicum* and *Gran. saccharobutylicum* was proved. Sera for two strains of *Cl. acetobutylicum* were produced; reciprocal cross and absorption tests proved them identical and one-way cross and absorption tests with 19 other strains of *Cl. acetobutylicum* in stock indicated them to be a remarkably homogeneous group serologically. *Cl. pasteurianum* and *Cl. felsineum* have given high specific titers but have shown no inclination to more than low group reactions with any of the other cultures tested. Other stock butyrics and other possibly less nearly related organisms, such as *Cl. multifermentans*, *Cl. bifermentans*, *Cl. tertium*, and *B. truffautii* have likewise given no indication of identity with any of the above chosen butyric cultures. Serologically then "the butyrics" are far from a homogeneous group. Correlation of their physiological and biochemical behavior with the serological data is essential. Such a study is in progress and until its completion we continue to use the species designations of the literature.

A9. A Comparative Study of Certain Presumptive Test Media. M.P.

HORWOOD AND ARTHUR HEIFETZ, Massachusetts Institute of Technology, Cambridge.

This inquiry was undertaken to ascertain the relative value of certain presumptive test media in bacteriological water analysis. Comparisons were made between standard lactose broth, crystal violet lactose broth, brilliant green lactose peptone bile, the Dominick and Lauter medium and the ferrocyanide citrate agar recommended by Tonney and Noble. Since the use of ferrocyanide citrate agar proved unsatisfactory, it was abandoned early in these experiments.

The comparisons were made by studying freshly isolated strains of *E. coli* from human feces, cow dung and horse manure, prepared as emulsions in sterile distilled water, and also by examining 50 waters, largely untreated, from a wide variety of sources. Stock cultures of *E. coli* and *Bact. aerogenes* were also used in the comparison. Inoculations were made using 10-, 1- and 0.1-cc. amounts of the water or the bacterial suspension in each medium. Ten tubes of each medium were used for each volume of water inoculated. Cultures were incubated at 37°C. and observed after 24 and 48 hours. Positive and doubtful presumptive tests were further identified by the partially confirmed and completed tests.

The results show that with freshly isolated fecal strains of *E. coli* prepared as an emulsion, standard lactose broth is the most sensitive of the four presumptive test media employed. Brilliant green lactose peptone bile is second, the Dominick and Lauter medium third, and Salle's crystal violet lactose broth, fourth. With untreated waters, the sensitivity of the presumptive test media corresponds to the order obtained when fecal strains of *E. coli* were used as bacterial emulsions. The positive presumptive tests obtained in brilliant green lactose peptone bile and in Dominick-Lauter medium when the waters were examined, gave the completed test for *E. coli* in 85 per cent of the tests, while only 70 and 72 per cent of the positive presumptive tests in lactose broth and Salle's medium could be completed. In view of the joint results obtained with fresh fecal strains of *E. coli* and the water analyses, we are of the opinion that standard lactose broth is better as a presumptive test medium than the other media used for comparison. Although some of the newer presumptive test media may yield a greater proportion of completed tests than standard lactose broth, their value is greatly diminished through their ability to inhibit strains of *E. coli* which have sanitary significance.

A10. *The Influence of Concentration of Soluble Calcium on the Precipitation of Calcium Carbonate by Microorganisms.* F. T. WILLIAMS AND E. B. FRED, University of Wisconsin, Madison.

It has been shown by several investigators that microorganisms are capable of precipitating calcium carbonate from artificial media containing soluble calcium salts. No attempt, however, has been made to determine the amount of calcium necessary in a solution in order that the microorganisms will precipitate the carbonate. Such information is desirable before any attempt is made to demonstrate the precipitation of calcium carbonate in the ocean or fresh water lakes.

Unidentified aerobic organisms from lake muds and several common aerobic organisms, such as *Ps. fluorescens*, *E. coli*, *Sarcina lutea*, *B. graveolens* and *Proteus vulgaris* are capable of precipitating calcium carbonate crystals on Molisch's glycerol-peptone-agar which contains 0.01 to 0.02 per cent soluble calcium as the chloride. Sea water contains approximately 0.04 per cent calcium in solution. The peptone used throughout these experiments was free of calcium. The criteria used in the detection of calcium carbonate in solid media were microscopic examination and effervescence of the crystals in contact with dilute hydrochloric acid.

In order further to demonstrate calcium carbonate precipitation by microorganisms, two known carbonate-precipitating organisms from lake muds were inoculated into Molisch's medium with varying amounts of calcium chloride present. The rate of carbonate precipitation was followed by periodic analyses of the cultures for soluble calcium. Crystals were separated from this liquid medium and washed. Chemical analyses showed these to be calcium carbonate with no traces of phosphate. X-ray analysis indicated that the carbonate was calcite. The x-ray pattern of these crystals is nearly identical with that of mud samples from the deposit of Lake Mendota.

A11. The Decomposition of Alginic Acid by Microorganisms. MELVIN C. ALLEN, New Jersey Agricultural Experiment Station, New Brunswick.

Polymannuronic acid or the so-called alginic acid was prepared from *Fucus vesiculosus* by extraction with dilute alkali solution and precipitation with dilute acid, followed by purification with chlorine dioxide. The purified material was subjected to decomposition by mixed, as well as pure, cultures of soil microorganisms. None of the fungi tested seemed to be capable of attacking this complex to any appreciable extent. However, certain bacteria were found to be capable of completely decomposing this complex. One organism was isolated in pure culture and used for further study.

In the decomposition of alginic acid by bacteria, the total uronic acid was found to decrease only slightly. The complex polymannuronic acid is completely precipitated with calcium chloride; however, the simpler form resulting from its decomposition is no longer precipitated by this salt. Neither does the simple form show the reducing properties of a primary uronic acid. The fact that the uronic acid content is not decreased appreciably indicates that the process is one of hydrolysis rather than of oxidation. The hydrolysis, however, seems to be incom-

plete, since no reducing compounds are produced. The decomposition is accompanied by a rapid increase in pH value of the medium, which may reach 9.0, depending on the rate of decomposition and the extent to which the medium is buffered.

A12. "Black Beets"—A Problem Involving Stimulation of Bacterial Growth by Iron. E. J. CAMERON, Research Laboratories, National Canners Association, Washington, D. C.

The occurrence of an abnormality known as "black beets" has, on widely separated occasions, caused loss to beet canners. Low vacuum of the container and a deep purple or intense black coloration of the entire contents of the can are evidences of this condition.

The reaction of the abnormal product is usually higher than pH 6.0 as compared with the normal pH of 5.3 to 5.4. Also, it has been found that in the abnormal product the amount of iron in solution may reach 40 to 50 p.p.m. as compared with a normal of 3 to 5 p.p.m.

During an investigation, which is still in progress, an organism has been isolated which is now regarded as the causal agent. This organism, which is a spore former, having an optimum growth temperature at 37° to 40°C., grows only moderately in plain beet juice and causes a slight increase in acidity. When, however, an iron strip is sterilized with the medium, thus bringing iron into solution in the beet juice, inoculation results in marked blackening of the medium. One effect of the iron is to increase the pH to 6.0 or slightly lower. Inoculation, however, results in a further increase in pH. This is usually to about 6.3 to 6.5 but has been observed to be as high as 7.2.

It has been shown that the stimulative effect of the iron is not related to the increase in pH that it brings about. Beet juice, or agar, partially neutralized to pH 5.8, will not darken without iron, nor will growth be markedly greater than that in the medium at pH 5.3.

In dextrose nutrient broth, growth of the organism results in production of acid. The reason for the apparently contrasting behavior in beet juice and in ordinary sugar media is obscure.

Colony structure varies greatly in different solid media. On plain nutrient agar, growth takes the form of thin, translucent, spreading colonies, which, in the earlier stages, are almost invisible. On tryptone agar, "droplet" colonies result. On iron-beet agar, "droplet" colonies also appear, sometimes conglomerating to form large transparent masses. Growth is abundant immediately over iron strips placed in the agar and especially so directly over the edges of the iron, whereas,

in the portions of the agar away from the iron, growth is very sparse or entirely absent.

A13. The Utilization of Slime-Forming Microorganisms. J. R. SANBORN, Research Division, International Paper Co., Glens Falls, N. Y.

The uncontrolled development of microorganisms in numerous industrial processes is generally detrimental to operative efficiency. The tendency for the majority of such forms to utilize the available nutrients in building up tenacious growths or slimes on appropriate surfaces of attachment, is one of the most troublesome features of the problem. In a pulp and paper system the slime-forming flora is widely diversified, consisting of bacterial species, alga-like forms, fungi related to the yeasts, and filamentous fungi.

In contrast to the undesirable and uneconomic aspects of slime development, it is quite possible that the activities of certain members of the group may be employed to advantage. Using special methods of cultivation, many of the causal species produce substances possessing desirable physical and chemical properties. For example, synthesis of polysaccharoses and other complex compounds through the action of various microorganisms on sugars, the formation of mucilaginous solutions, and the gelatinous condition produced in suspensions of cellulose by numerous species of fungi, are among the reactions which have a definite bearing upon the utilization of fibrous cellulose.

The characteristics of the materials formed by the members of the slime group are highly variable. These include the soluble substances which are more or less closely related to the gums as classified tentatively by Haas and Hill, and also the insoluble, cellulose-like products built up by a number of organisms. Substances are formed, as well, which are intermediate in their relationship to the former classes; these resemble the so-called oxy- or hydro-celluloses. The adhesive and cohesive qualities of these complex mixtures of slimes are revealed in the continuous films and membranes which are readily formed when slime particles in suspension are allowed to coalesce.

A14. Bacteriological Studies of the Red Discoloration of Salted Hides.
A. G. LOCHHEAD, Division of Bacteriology, Central Experimental Farm, Ottawa, Canada.

A study was made of organisms concerned with the red discoloration of salted hides, also termed "Red heat," which defect may occasion con-

siderable loss in the leather industry through spotting and weakening of the fiber. Red sarcinae, strongly halophilic and similar in type to forms described by Lloyd et al., from discolored hides cured with marine salts, were isolated from Argentine hide. On Canadian hides showing red discoloration the active agents were found to be pleomorphic motile rods representing two species.

Discoloration on salted cowhides was found to be due to an organism closely related to *Serratia salinaria* (Harrison and Kennedy) Bergey et al., the cause of red discoloration of cured codfish in Eastern Canada. From salted buffalo hide from Western Canada showing discoloration an organism designated as *Serratia cutirubra* (*Bacterium cutirubrum*), nov. sp., was isolated as causal agent. Like *S. salinaria*, it is an obligate aerobe and halophile. It develops in a NaCl range of 20 per cent to saturation, with optimum growth at 28 per cent. It is likewise Gram-negative and motile and highly pleomorphic, varying from long rods in young to coccoid forms in older cultures, with intermediate forms. It forms a deep red color on salted fish and milk media, and on the latter and on salt gelatine shows more marked proteolytic action than *S. salinaria*. Through their proteolytic effect these organisms are considered capable of greater damage to hides than the red sarcinal types which are non-liquefying.

An agar medium of 50 per cent skim milk with 24 to 28 per cent salt proved a simple and effective substrate for the isolation and cultivation of the rod forms. It was selective in excluding almost entirely halophilic non-chromogens and red cocci. These types could be isolated with a peptone, beef and yeast extract agar with 16 to 20 per cent salt, which medium in turn excludes the red halophilic rods.

Red, halophilic, non-liquefying sarcinae, though present on some Canadian hides, are not considered important in producing the red discoloration. The non-chromogenic halophiles isolated were mostly liquefying rods developing in a salt concentration range of 10 to 24 per cent. Whether they are active in causing damage through association with the red halophilic rods has not been determined.

A15. An Apparatus for the Control of Composition and Rate of Flow of Gas Mixtures Through Culture Solutions. C. E. SENSEMAN,
U. S. Department of Agriculture, Washington, D. C.

This apparatus was constructed originally for the purpose of studying the production of citric acid from glucose by *Aspergillus niger* under variable atmospheric conditions. Its efficiency of operation has led to

its utilization in other investigations now in progress. The two gases to be mixed are conducted separately to manifolds, each having 6 needle valve outlets. Six gas variables thus may be studied simultaneously. One flowmeter placed after each manifold measures the volume of that gas per minute coming through each valve. Following this measurement, the two gases are brought together and passed through a humidifier. This gives the desired composition for each of the 6 mixtures. The mixed gas in each line then passes to its own manifold, which has 4 needle valve outlets, each joined to a flask in a constant temperature box. Twenty-four flasks are thus running at one time. The outlets of the flasks are all connected to a glass manifold which has another opening to a single flowmeter. By adjusting any one needle valve leading to its respective culture flask, the desired volume of gas for that flask is readily measured. After adjustments, the effluent gases from individual flasks are shunted, by means of stopcocks, from the flowmeter either to the open room through short capillary tubes or to individual carbon dioxide absorption tubes. The space required for the apparatus is approximately 7 feet long, $3\frac{1}{2}$ feet high, and 2 feet wide.

A16. *The Fermentation of Citron.* C. R. FELLERS AND E. G. SMITH, Massachusetts State College, Amherst.

The citron, *Citrus medica* Linn., which is imported in brine from Southern Europe, is now in limited commercial production in Puerto Rico, Florida and California. In Corsica, the unripe citron is cut in half, fermented in two changes of sea water for 40 to 50 days, and salt added to make a 10 to 12 per cent concentration for export. The fermented citrons are clear, translucent, and have a very pleasing aromatic odor. Fermentation studies in 5 per cent salt brine on Puerto Rican and Florida citrons confirm the findings of Hollande and Chade-faux (Bul. sci. pharm., **31**, 458-471; 527-539) relative to the microorganisms concerned in the fermentation of Corsican citrons, namely *Saccharomyces citri medicae* and *Bacillus citri medicae*. The cultural, morphological and biochemical characters were identical. The organisms were present on the unripe fruits. Scum yeasts, which were abundant in the brine after a few days fermentation, took no part in the fermentation, but destroyed the acids and caused spoilage unless controlled. A typical fermentation yielded 7 grams of acid (calculated as acetic acid) per liter after 14 days. The rise in acidity paralleled the increase in numbers of bacteria and yeasts. Bacteria reached their maximum numbers in 20 days and yeasts in 10 to 15 days. The bacteria do not resemble the lactobacilli present in fermenting cucumber pickle brines.

A17. Production of Agglutinins against Thermophilic Organisms. L. S. McCLUNG, University of Wisconsin, Madison.

During the course of an investigation concerning the classification of strains of thermophilic, aerogenic anaerobes causing swells in canned foods, it was found that the physiological characteristics of the various strains were not sufficiently diagnostic to warrant the conclusive separation into species, thus making desirable serological data. Search of the literature failed to reveal extensive use of immunological results with thermophilic organisms.

A procedure is described which has given satisfactory results in the production of an anti-serum against anaerobic, spore forming, thermophilic organisms. Sera obtained after 5 intraperitoneal injections of fresh unwashed cells given at 3 day intervals, followed by a wait of 6 days, have given specific titer readings in the range of 1:1280 to 1:5120. Beginning the ninth day after the last intraperitoneal injection, each animal received an additional treatment of 3 daily intravenous injections. No marked rise in titer was produced by this treatment. The usual antigen-serum dilutions were made and the routine procedure of incubation has been for 4 hours at 52° to 54°C. Following overnight refrigeration, readings were taken against a single source of light in a darkened room. After direct and cross agglutination studies were made, the absorption technique was attempted. Successful results were obtained using a concentrated antigen as above. The serum and dilute suspensions of antigen used for the tests were preserved by the addition of 0.2 per cent phenol and stored in the ice box. Both remained stable over the period of 4 months during which the tests have been made.

The serological and physiological data, together with a satisfactory technique of cultivation of these organisms, will be published.

A18. The Microbiology of Canned Meat Products. E. H. RUYLE AND F. W. TANNER, University of Illinois, Urbana.

Since meat products are as susceptible to bacterial attack as are other perishables preserved in sealed containers, the possibility of the presence of microorganisms capable of producing spoilage or in some way rendering the food injurious to health, has been considered. The following canned products, secured from commercial stocks, were examined: (a) 379 cans of various commercially canned meat products examined as soon as possible after receipt; (b) 199 similar products examined after 3 to 6 months storage at room temperature; and (c) 43 cans regarded with suspicion and sent to the laboratory for immediate bacteriological analysis.

Bacteriological data secured in this investigation supports the opinion that microbes present on meats occasionally survive the process. The fact that a few suspected cans supposedly sterilized, actually spoiled, is further evidence of occasional underprocessing. The percentage of merchantable cans containing organisms as revealed by this study is 5.5 for products other than spiced ham. The latter product, processed at temperatures below 212°F., was found to carry viable organisms.

Although the aerobic organisms recovered indicate an insufficient process there is as yet little evidence that they play a rôle in spoilage of canned meat products. The obligate anaerobes isolated in this investigation, however, putrefied protein media under proper anaerobic conditions. Their potential danger, therefore, must be realized regardless of the fact that they were occasionally isolated from apparently normal samples. Although anaerobic enrichment cultures proved to be non-toxic upon feeding to guinea pigs, the possibility of *Clostridium botulinum* surviving the process must also be considered.

Viable organisms were not recovered from any of 46 containers found to be defective (496 cans were examined by experts in can manufacture) indicating that leaky containers are by no means always infected. Bacteria found in this study, therefore, allowing for possible contamination were caused either by underprocessing, an unusually heavy bacterial load, or to other factors needing further study.

A19. Use of the Spiral Absorber for the Determination of Carbon Dioxide.

HARRY E. GORESLINE, U. S. Department of Agriculture,
Washington, D. C.

The determination of carbon dioxide is often used as a means of judging the progress of fermentations and other biological processes. A spiral absorber of the Hahn type is well adapted to the absorption and determination of the carbon dioxide evolved from such processes. This absorber consists of a 4-mm. glass tube, approximately 15 feet in length, wound into a close spiral and sealed to a gas delivery tube incorporating a bubble "pump." The glass spiral is inserted into a large test tube which holds the absorbing liquid, and as each bubble of gas ascends the spiral a small quantity of the absorbing liquid is "pumped" in behind it. In this way the gas is held in direct contact with the absorbing solution until all of the CO₂ has been removed. Potassium hydroxide is used as the absorbing medium.

This absorber is simple, easy to manipulate, and is especially useful in rapid routine determinations. The apparatus may be operated either by drawing CO₂-free air through the test material to sweep the evolved

gas into the absorbing medium, or by allowing the pressure developed during fermentation to force the gas through the absorber. Tests have demonstrated that CO₂ concentrations up to 95 per cent are absorbed completely by the absorber up to its full capacity of approximately 80 cc. per minute. Titrations on the absorbing medium agree favorably with the absorption bulb weighing method. This apparatus has been used in connection with the CO₂ determination on various biological materials and under different conditions and has proved very satisfactory.

A20. The Numbers and Types of Bacteria Surviving in Household Dusts after Storage in Sealed Containers for Two Years. M. P. HORWOOD, B. S. GOULD AND H. SHWACHMAN, Massachusetts Institute of Technology, Cambridge.

In 1930, a paper was presented before this Society summarizing the results of an investigation into the bacteriology of 47 samples of household dusts. Since then, the same dusts had been kept in sealed Atlas preserving jars equipped with rubber rings and tightly fitting glass covers. The samples were stored at room temperature in the dark for two years. The present study is a summary of the effects of storage under the conditions described on the bacterial content of the same household dusts. Identical methods of examination were used in both inquiries, so that the results would be strictly comparable.

The total count on nutrient agar at 20°C. after 5 days incubation showed marked reduction. While only 9 samples of fresh dust showed counts under 500,000 per gram, 46 stored samples yielded results in this category. The average count diminished from 3,500,000 per gram to 196,000 per gram, a reduction of 94.3 per cent. At 37°C. after 48 hours incubation, the average count per gram of dust diminished from 4,200,000 to 140,000, a reduction of 96.6 per cent. Only 8 samples out of 46 fresh dusts showed counts under 500,000 on nutrient agar at 37°C. after 48 hours, whereas 44 stored samples out of 45 examined showed counts under 500,000. Furthermore, the ratio of the 20°C. to the 37°C. count increased from 0.833 in the fresh dusts to 1.40 in the stored dusts, showing a more rapid destruction of body than soil forms.

While gas formers in lactose peptone bile were absent in 3 out of 46 samples of fresh dust, 28 of the 47 stored dusts showed a complete absence of lactose fermenting organisms. In the remaining 19 samples, the concentration of gas-forming bacteria was greatly diminished. Similarly, acid-forming bacteria on purple lactose agar at 37°C. after 48 hours were uniformly absent, but were present in abundant numbers in

32 of the 47 original samples. The presence of overgrowths in the other 15 samples prevented an accurate determination of the concentration of acid formers in these samples.

Aerobic spore-formers, present abundantly in the fresh household dusts, were somewhat diminished on prolonged storage, but were still present in large numbers. Spore-forming bacteria survived to a greater degree than any other type.

The total count on blood agar at 37°C. after 2 days, incubation showed a marked reduction in the stored samples. While only 6 out of 46 fresh dusts showed counts on blood agar under 250,000 bacteria per gram, 43 out of the 47 stored samples showed a total count less than this amount. Similarly, while hemolytic bacteria were absent in only 3 samples of fresh dust, negative results were obtained in 40 dusts or 85.2 per cent of the samples after storage. Again, while only 9 of the fresh dusts, or 20.4 per cent, showed an absence of streptococci in 0.5 gram samples that were incubated in meat infusion nutrient broth for two days at 37°C., 42 or 89.4 per cent of the stored samples showed an absence of streptococci when examined in this way.

In the original study, 255 hemolytic and 147 non-hemolytic colonies were examined by making smears, staining and examining microscopically. Hemolytic streptococci were found in 20 out of 45 samples of dust and non-hemolytic streptococci in 14 of the dusts. Either or both types of streptococci were found in 60 per cent of the dusts examined. The examination of the same smears showed the presence of hemolytic staphylococci in 21 out of 45 samples and non-hemolytic staphylococci in 27 samples. Considered together, staphylococci were present in 33 samples or 73 per cent of the fresh dusts. In the examination of the stored dusts, 299 smears from colonies obtained on blood agar plates were examined, and of this number none showed the presence of streptococci and only one, the presence of staphylococci.

There was a slight loss in the moisture content of the dusts after storage, but since the fresh dusts all contained less than 6.0 per cent moisture, the loss on storage could not be appreciable.

A21. An Improvement in the Methylene Blue Reduction Test. L. A. BURKEY, U. S. Department of Agriculture, Washington, D. C.

An intensive study has been carried on for several years in an effort to modify the methylene blue reduction test so that a quicker and more accurate method could be developed for indicating the bacterial content of high grade milk.

The results of this work have shown that a number of substances can be added to the milk-methylene blue mixture which will stimulate the growth of bacteria during the test and thereby shorten the reduction time. The addition of 0.1 to 0.5 per cent of peptone to the milk-methylene blue mixture at the beginning of the test shortens the reduction time as much as 25 to 30 per cent as compared to the results obtained where no peptone is added. The addition of peptone has very little effect on the oxidation-reduction potential of the milk or on the absorption of oxygen from the milk. Its whole function seems to be the stimulation of bacterial growth, particularly that of *Streptococcus lactis*. The relative number of lactic and of gas-forming types and the activity of each are factors which determine the efficacy of the peptone addition as well as uniformity in results when the provisional method is used. A scarcity of *Strep. lactis* in the milk increases the reduction time. However, in over 200 milk samples, decidedly better correlation is shown between reduction time and total count when peptone is added than when the usual methylene blue reduction test is used.

A22. Lipolytic Activities of Several Bacteria Causing Bitter Cream.

JAMES T. McGRATH AND J. A. ANDERSON, Rutgers University,
New Brunswick, N. J.

This work is on the cause of bitter flavors in cream. The bacteria studied were described in a paper by Anderson and Hardenbergh presented at the 1931 meeting of this Society. The only significant change produced by these organisms is their ability to hydrolyze fats, which they do with reasonable rapidity. The first biochemical studies were concerned with the rates of acid production in cream (due to hydrolysis of butter fat) at various temperatures. Determinations were made by titration, in neutral alcohol, and by determination of unhydrolyzed fat after various time intervals. Later the changes on pure triglycerides of fatty acids occurring in butter fat were studied. It was found that only the glycerides of the fatty acids of lower molecular weight, with the exception of tripalmitin, were rapidly hydrolyzed; of these tributyrin was most readily attacked. The glycerides of the semi-solid and solid fatty acids were altered little in 25 days with the exception of tripalmitin which appeared to be attacked slowly, but which once attacked underwent change fully as rapidly as tributyrin.

Addition of very small quantities of certain of the fatty acids of low molecular weight to cream developed tastes, odors, and throat irritations very similar to those encountered in certain types of bitter cream pro-

duced naturally or with pure cultures. The acids most marked in their ability to produce these effects were caproic and isocaproic acids. Caprylic acid produces a similar effect but the odor is less sharp and more rancid and tallowy, with a bitter taste.

A23. An Agar Plate Method for the Detection and Enumeration of Lipolytic Microorganisms. J. A. ANDERSON, Rutgers University, New Brunswick, N. J.

In the course of investigations of milk and cream spoilage due to lipolytic bacteria the development of a medium which would detect such microorganisms became highly desirable. A number of media, most of them synthetic, containing various natural and modified oils and fats, were tried out. None was entirely satisfactory. But one which served the purpose admirably was prepared by adding approximately one per cent by volume of neutral tributyrin to nutrient agar. This medium when plated is turbid because of the small globules of tributyrin. Conspicuous clear zones develop around lipolytic colonies, the width of the zones varying with different organisms. The addition of such indicators as brom thymol blue, phenol red, and brom cresol purple, using 7.5 cc. of 0.5 per cent alcoholic solution of the indicator, enhances the value of the medium, for lipolytic action is accompanied by changes in reaction. Since some bacteria produce shifts to an alkaline reaction and others to the acid side, it is thought the medium should be approximately neutral, but none of the many lipolytic organisms isolated appear to be appreciably sensitive to changes in reaction. It is advantageous to prepare the medium in 100-cc. portions in 250- or 300-cc. Erlenmeyer flasks and to pour plates directly from the flask. But little shaking is necessary to secure a fairly uniform distribution of the fat. This medium has been used in the examination of milk, cream, rinsings from dairy equipment, water and sewage. It appears to have a wide range of usefulness. The medium is highly selective. Few non-lipolytic bacteria will develop on it.

A24. The Acidoproteolyses in Gaseous Associative Fermentation in Milk.
COSTANTINO GORINI, Agricultural High School, Milan, Italy.
[Read by title.]

The phenomenon of gaseous associative fermentation is characterized by the gas production which is brought about by the symbiotic or synergic action of different organisms on certain carbon compounds, although none of the organisms is able to bring about such changes by itself.

This phenomenon, first noted by Kendall in milk (1910), was afterwards studied in artificial media by Holman (1914) and many others who concluded that it occurs when there is the association of two saccharolytic bacteria, if one of them is capable of producing acid (never gas) from a carbon compound present in the culture medium, and the other is capable of producing gas from another carbon compound (e.g., glucose). Ishikawa (Jour. Inf. Dis., 1927, **41**, p. 238) found that in milk the association of saccharolytic organisms is not sufficient, but that the association of two saccharolytic organisms with a proteolytic organism is necessary. He thinks that the phenomenon occurs in artificial media when only two saccharolytic bacteria are present, because those media already contain products of proteolysis (peptone, amino acids, etc.).

I have repeated the researches of Ishikawa and confirmed his results; but I found besides that for the production of the phenomenon in milk the association of the three mentioned organisms is not always necessary; even two saccharolytic bacteria may be sufficient, provided one of them belongs to the group of *Acidoproteolytes* Gorini which are not only saccharolytic but also proteolytic, like many strains of *Mammococcus* Gor., *Streptococcus*, *Enterococcus*, *B. coli* and other bacteria which commonly are classified among the simple saccharolytic bacteria (Gorini: Rend. R. Acc. Lincei, 1920, **29**, p. 114; Rend. R. Ist. Sc.; Lett. 1923, **56**, p. 994 and 1927, **60**, p. 644).

A25. The Effect of the Temperature of Incubation Upon the Agar Plate Count of Milk. CARL S. PEDERSON AND M. W. YALE, New York Agricultural Experiment Station, Geneva.

The observation that 14 different bacteriological incubators varied at least 2°C. during a 48-hour period, even when lightly loaded with agar plates, led to a study of the effect of the temperature of incubation upon the standard agar plate count from various dairy products. Series of duplicate agar plates were placed in incubators operating at temperature intervals between 21° and 55°C. Average colony counts were calculated as percentages of the maximum average colony count and plotted against temperatures of incubation. A smooth curve was constructed from which by interpolation, the equivalent percentage of the maximum colony count for any temperature of incubation could be determined.

Studies were made from 78 samples of pasteurized and raw milk, cream and ice cream secured from various sources in 13 cities including Boston, New York, Detroit and Philadelphia.

An incubation temperature of 32°C. for 48 hours instead of 37°C. is recommended for standard agar plates prepared from samples of milk and ice cream for the following reasons: (1) With 48-hour incubation 37° colony counts averaged approximately 50 per cent of the 32° counts. (2) At 32°C. the error in colony counts due to temperature variations of 1° averaged less than 3 per cent, while at 37° they averaged approximately 25 per cent. (3) The 37° count of the normal samples of pasteurized milk ranged between 10 and 89 per cent of the maximum, while the 32° count ranged only between 85 and 100 per cent of the maximum. Therefore, the latter temperature serves as a better means of comparing the quality of different samples.

A26. The Composition of Standard Media for Use in Routine Milk Control Work. CHESTER S. BOWERS AND G. J. HUCKER, New York Agricultural Experiment Station, Geneva.

The medium as suggested by the American Public Health Association for the Standard Methods of Milk Analysis has been subjected to considerable criticism. This criticism has been based largely on the experience of those who find that it does not promote the growth of all of the organisms commonly found in milk. A study was undertaken to determine the relative efficiency of this medium in comparison with other media. In addition, an effort was made to enhance its efficiency by varying certain of its constituents.

A large series of samples of raw and pasteurized milk was studied. Comparative counts were secured using standard medium, together with a series of media so adjusted as to determine the effect of the various constituents of standard medium upon its efficiency. In addition, these variations of standard media were compared with a medium which contained in addition to standard media, tryptophane broth peptone, dextrose and yeast extract.

The results of these investigations indicate that the substitution of yeast for beef extract did not enhance the efficiency of the medium. The addition of a fermentable carbohydrate generally caused an appreciable increase in the number and size of the colonies. The addition of tryptophane broth peptone, glucose and yeast extract to standard media increased the counts secured about 4 times. It is suggested that standard media be so adjusted that it serve as a more accurate index to the number of organisms actually present.

- A27. *A New Species Belonging to the Genus Bacillus*. HARRY E. GORESLINE, U. S. Department of Agriculture, Washington, D. C.

During studies on a trickling filter receiving a creamery waste, a bacterial culture was isolated whose morphological and physiological characteristics indicated that it was a new species belonging to the genus *Bacillus*. This microorganism has the following general characteristics: A Gram-positive (later becoming Gram-negative) slender rod occurring in long chains, motile, producing terminal spores and exhibiting granular staining with most of the standard dyes. It produces rarely small bud-like cells on the end of certain rods. Surface colonies on agar are white, rhizoid and spreading, while subsurface colonies present a nebulous appearance, containing many knots and swirls in the hazy growth. Because of this nebula-like appearance the name *Bacillus nebulosus* nov. sp., has been given to this microorganism. Gelatin is liquefied, nitrates are reduced and dextrose, levulose, maltose, salicin and dextrin are fermented with the formation of acid, but without gas. Indol is not formed. This culture was a part of the Iowa Engineering Experimentation Culture Collection.

- A28. *Studies on a Herd Infected with Brucella Abortus. II. Incidence of Milk Infection in a Vaccinated Herd*. DOROTHY W. CALDWELL, NEIL J. PARKER AND EDGAR M. MEDLAR, HEGEMAN MEMORIAL RESEARCH LABORATORY AND THE FARM OF THE METROPOLITAN LIFE INSURANCE COMPANY SANATORIUM, MOUNT MCGREGOR, N. Y.

Herd studies over a period of three years showed that 23.6 per cent of the reactors were discharging *Brucella abortus* in their milk.

Irregularity in the incidence of *Brucella abortus* in the milk of vaccinated cows led to an intensive study of the milk of reactors from which this organism had not been isolated. Thirteen cows were selected. Two hundred and eighty-eight daily, followed by 51 monthly samples gave negative results.

Agglutination studies of the milk sera of 21 blood serum reactors were made. Two hundred and fifty-eight samples of milk from these cows had been inoculated into guinea pigs with negative results. Milk serum agglutination tests were negative for all but one cow.

This cow was studied intensively. Daily tests of milk from each quarter were made for a period of four weeks before calving. These were negative, except for milk from the right rear quarter which had a titer of 1:120. Guinea pig inoculations gave negative results with the

exception of one animal which had received milk from the right rear quarter near the end of the milking period. After the cow had calved two series of daily milk samples, of two weeks each, were examined. Agglutinins were found in samples from all four quarters, the titer varying from 1:120 to 1:240 in the three quarters previously negative, to 1:960 in the quarter which had been positive before calving. *Brucella abortus* was repeatedly isolated from milk of both right quarters but was not found in samples from either left quarter.

A29. *Brucella melitensis* Infection in Cattle. R. A. BOAK AND C. M. CARPENTER, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

We previously reported the results of a survey of *Brucella abortus* infection in raw milk from 3 counties in central New York. Of 122 samples 20.4 per cent showed by guinea pig injection evidence of the infection. Three of the cultures proved to be *Br. melitensis* upon further serological and biochemical examinations and by monkey inoculation. These cultures were isolated from samples of milk collected from herds in 3 rather widely separated towns in the area surveyed. Possible contact with goats was determined in 2 instances. Experimental data on the injection of cattle with cultures of *Br. melitensis* are submitted showing their relative resistance to the strains used. A brief history of this infection in cattle in the United States is given.

A30. *The Bacteriology of Chronic Mastitis.* G. J. HUCKER AND P. ARNE HANSEN, New York Agricultural Experiment Station, Geneva.

A résumé has been prepared of the investigations on udder infections carried on at the New York State Agricultural Experiment Station. During the past 5 years over 125 autopsies have been performed and the results correlated with a laboratory examination of the milk secured prior to post mortem. In addition, regular quarter samples have been examined from over 200 cows over a period of two years. The history of each individual has been followed by cooperating clinicians.

The results of these studies indicate the prevalence of chronic or subclinical mastitis as well as the significance of the laboratory examination of milk in the detection of abnormal udders.

A detailed study has also been completed pertaining to the types of streptococci present in the udder and the relation of leucocytes in the milk to the presence of udder infection.

A31. Observations on Organisms Associated with Chronic Bovine Mastitis.

W. N. PLASTRIDGE, E. R. SPAULDING AND G. D. BRIGHAM,
Storrs Agricultural Experiment Station, Storrs, Conn.

As part of a research program on bovine mastitis, which was begun in 1926, periodic laboratory examinations have been made on milk from individual quarters of 180 animals distributed in 4 commercial dairy herds. Tests were made on 2 herds over a period of 5 years, and on the other 2 herds over a period of 2 years. For the purpose of securing information on the organisms responsible for chronic mastitis, repeated isolations of the predominating organisms occurring in the milk of animals with a definite history of mastitis have been made. The data obtained indicate that of 49 udders affected with chronic mastitis of from one month to 5 years duration, 35 were infected with streptococci and 14 with staphylococci.

The streptococcus strains were divided into 9 groups on the basis of ability to produce acid from mannose, mannite, raffinose, inulin, sorbitol, salicin and trehalose. All strains fermented dextrose, galactose, lactose, levulose, maltose and sucrose and failed to ferment arabinose, dulcitol and xylose. On repeat tests, it was found that certain strains lost the ability to produce acid from trehalose and others gained this property. Results obtained with glycerine were generally unsatisfactory. Disregarding the reactions obtained with trehalose and glycerine, 77 per cent of the streptococcus strains fell into a single group. The different groups could not be distinguished by any of the following tests: Colonies on blood agar, hemolysis in blood broth, hydrolysis of sodium hippurate, reduction of methylene blue milk, reaction in litmus milk, and final pH in dextrose broth.

Forty-nine strains of staphylococci obtained from chronic cases of mastitis were divided into two groups on the basis of ability to form acid in salicin broth.

MEDICAL BACTERIOLOGY, IMMUNOLOGY AND
COMPARATIVE PATHOLOGY

M1. Etiology of Encephalitis in St. Louis, 1933, and Its Differentiation by Protection Tests. LESLIE T. WEBSTER AND GEORGE L. FITE, Rockefeller Institute for Medical Research, New York, N. Y.

Four strains of virus from brain tissue of fatal cases of St. Louis encephalitis and one strain from a similar Kansas City case have proved

serologically identical. These strains have been compared with the Muckenfuss, Armstrong, McCordock virus.

Monkeys given 2 injections of our virus develop in their sera specific protective bodies. Sera from convalescent cases of St. Louis and Kansas City encephalitis possess similar protective bodies. Sera from convalescent cases in Paris, Ill., Cincinnati, Ohio, and Indianapolis, Ind., have likewise been tested. Finally, sera from New York cases of so-called epidemic encephalitis—acute, recent convalescent, and chronic types, with and without sequelae—have been studied for the presence of the specific protective bodies.

The findings to be reported bear on the questions of the nature of certain human encephalitides and the relationship of epidemics and cases appearing at different places and at different times.

M2. Specificity of the Protection Test in Yellow Fever. W. A. SAWYER
AND LORING WHITMAN, International Health Division, Rockefeller Foundation, New York, N. Y.

The intraperitoneal protection test in yellow fever (Sawyer and Lloyd) is probably unique among immunity reactions in the large scale on which it has been applied to the sera of persons known to have been free from previous exposure to yellow fever virus or to have been definitely infected. The greater proportion of over 12,000 sera tested in New York were, however, from persons of unknown yellow fever experience. The countries in which there is reason to believe yellow fever has never existed, either on account of their medical history or the absence of the usual mosquito vector (Canada, China, Philippine Islands, Malaya, India, Egypt, Abyssinia, the East African Coast, South Africa), sent shipments of sera which usually included no protective sera whatever. Taken as a group, less than one per cent of the sera from these countries showed protective power. The rare protective sera might be explained as coming from persons who had been actually exposed to yellow fever contrary to the history given, or as being due to inaccuracies in testing or to hypothetical cross immunity or non-specific immunity. In our experience, in all cases in which tests could be made, known yellow fever or vaccination against yellow fever has invariably been followed by the acquisition of demonstrable protective power in the serum. We conclude that the intraperitoneal protection test is highly specific and affords strong evidence as to the presence or absence of previous infection with yellow fever virus, and is especially reliable with regard to infection in recent years.

M3. Studies of Circulating Virus and Antibodies in Yellow Fever Infection in Animals. T. P. HUGHES AND MAX THEILER, International Health Division, Rockefeller Foundation, New York, N. Y.

When susceptible monkeys are inoculated with unmodified yellow fever virus the virus appears in the circulation after an incubation period of one to 10 days and this incubation period increases with a decreased dosage of virus. Then follows a rapid increase in circulating virus, the rate of increase being independent of the inoculating dose. When insusceptible monkeys, or mice, are similarly inoculated the virus is taken immediately into the circulation, and disappears in the course of 2 days; the rate of absorption and elimination parallels closely the rate of absorption and elimination of an inert protein such as ovalbumin.

When susceptible monkeys are inoculated with large doses of yellow fever virus "fixed for mice," the virus is absorbed immediately and circulates for one or 2 days, shortly after which time protective antibodies appear in the circulation. The rate of absorption and elimination in this case also parallels that of ovalbumin. When minute doses are inoculated, no virus is demonstrable in the circulation for a period of 2 to 4 days; it is then present in the circulation for one to 3 days, after which time protective antibodies appear. In the case of mice, it has been demonstrated that the degree of immunity resulting parallels the quantity of virus inoculated.

Two types of antibodies may result from yellow fever infection. The protective antibody results from the presence of virus itself, and is formed whenever the virus is present in adequate concentration, regardless of the occurrence of demonstrable evidence of infection. A precipitating antibody occurs subsequent to severe infections only and probably reflects the response to products of cell destruction.

M4. Properties of Vaccine Cultivated in the Chorio-Allantoic Membrane of Chick Embryos. E. W. GOODPASTURE AND G. J. BUDDINGH, Vanderbilt University Medical School, Nashville, Tenn.

A technique is described for inoculating the chorio-allantoic membrane of 10- to 12-day embryo chicks with vaccinia virus, and for harvesting the vaccine free of bacteria and producing it in quantity. The vaccine may be preserved fresh, glycerinated or dry for long periods without appreciable loss of virulence.

The vaccinal lesion of the membranes is described and discussed. The 3 germinal layers of the chorio-allantois become infected and the yield of vaccine is large. Guarnieri bodies and Borrelia (Paschen

bodies) are abundant. Demonstration of the latter is used as an index of potency.

A dermal strain of vaccinia virus has been maintained in the embryonic membranes during the past year and a half through 100 successive generations without intervening mammalian passage. This strain has apparently become stabilized in its virulence for the chick embryo, for rabbits and, so far as preliminary experiments have gone, for man.

Thirty-six persons have been vaccinated with various generations of the chick vaccine, including the latest or one-hundredth generation. These have been controlled by similar vaccinations with potent calf vaccines.

The chick vaccine induces local lesions and general responses quite comparable with those induced by calf vaccine. The former are in general milder but run a course of equal duration and extent. Those vaccinated with chick vaccine have been revaccinated with calf vaccine and only immune reactions have resulted.

Serum from persons vaccinated with calf vaccine and from those vaccinated with chick vaccine show a strong and equal antiviral effect when titrated individually on the rabbit's skin.

Before final conclusions relative to the immunizing effect of the chick vaccine in the human can be drawn, it will be necessary to observe the relative duration of immunity in two series of cases, the one vaccinated with calf vaccine, the other with chick vaccine, over a considerable period of time. Selected groups from each series at intervals will be revaccinated with calf vaccine and the results compared both from the standpoints of local response, and serological changes.

For this purpose there has been placed at our disposal a group of people who may be observed under like conditions over a long period of time.

M5. A Comparison of the Antigenic Qualities of Killed and Living Vaccine Virus in the Normal Rabbit. JAMES CRAIGIE, University of Toronto, Toronto, Canada.

Following inoculation with living vaccine virus, agglutinins, precipitins and complement fixing antibodies for the elementary bodies and the specific precipitable substance of vaccinia make their appearance in the serum of the rabbit about the eighth day. This report deals with the formation of these antibodies in the normal rabbit when killed elementary body suspensions are injected.

The suspensions of elementary bodies employed for injection were

obtained from the rabbit. They were washed 3 times in the angle centrifuge to remove free specific precipitable substance. After purification the elementary bodies were killed, either by formalin, by boiling or by prolonged contact with ether at 37°C. The treated suspensions were then tested by testicular injection and two subsequent serial testicular transfers to exclude the presence of living virus. During the course of the experiments the rabbits were isolated in special quarters and strict precautions were taken to guard against importation of living virus. Three injections of killed elementary body suspension were given intravenously, subcutaneously or intradermally at intervals of 9 to 11 days. The macroscopic method was used in testing the sera for agglutinins and precipitins, formalized elementary bodies and virus-free Seitz filtrates, respectively, being used as antigen in these tests. Ice-box fixation followed by incubation at 37°C. for one hour was employed in the complement fixation tests.

In rabbits inoculated intravenously with one or other of the killed elementary body suspensions agglutinins appeared after the first injection, but 2 or 3 injections were required before complement fixing antibodies were demonstrable and 3 were required before precipitins made their appearance. Formalin-killed elementary bodies caused a greater antibody response than heat-killed elementary bodies, but the difference was quantitative rather than qualitative. High agglutinin titers (average 1:1000) were obtained with 3 intravenous injections of formalin-killed elementary bodies. Agglutinin titres in rabbits vaccinated with living virus were definitely lower (average 1:300). Intravenous injection of virus-free filtrate containing the specific filterable substance of vaccinia produced a response similar to that evoked by heat-killed elementary bodies.

Subcutaneous and intradermal injection of formalin-killed elementary bodies yielded poor responses, the agglutinin titres being low, complement fixing antibody appearing only in some animals and precipitins being absent. Ether-killed elementary bodies were definitely inferior to formalin-killed elementary bodies.

With the exception of the formation of agglutinins when formalin-killed elementary bodies are injected intravenously, the delay or absence of response in normal rabbits to killed elementary bodies contrasts with the prompt formation of agglutinins, precipitins and complement fixing antibodies which occur when living vaccine virus is applied to the skin of the normal rabbit.

M6. Age, Breed and Species Susceptibility in Transmissible Leukosis.

E. L. STUBBS, University of Pennsylvania, Philadelphia.

A description is given of experiments conducted with transmissible leukosis in day old chicks, chicks up to 2 months of age, young chickens, 4 months to one year of age, and old chickens, 2 to 4 years of age. The shortest periods of incubation with an average of 19 days, occurred in baby chicks. The highest incidence of successful transmissions occurred in baby chicks with every chick becoming affected in some groups. The highest mortality was in the baby chicks. The younger the chicken, the shorter the period of incubation, the more numerous the takes and the higher the mortality, which shows that the younger the chicks the more susceptible. All ages became affected with transmissible leukosis.

Six groups of different breeds of chickens were injected with transmissible leukosis, including Barred Plymouth Rocks, White Leghorns, Rhode Island Reds, Mixed Mongrels, Bantams and Naked Necks. Members of each group became affected. There was some variation in the incidence and mortality in the various groups. The fact that members of each group became affected, indicates that all breeds of chickens are susceptible to transmissible leukosis.

Groups of guinea fowls, turkeys, pheasants, pigeons, ducks and geese were selected to try out the species susceptibility. Members of each group were injected with transmissible leukosis. None of the members of any such group became affected. One pheasant-chicken hybrid was obtained and injected. This bird sickened and died with transmissible leukosis. Before death, attempts were made to transfer leukosis from the hybrid to pheasants but without success. Attempts to transmit leukosis to birds other than chickens were unsuccessful. This strain of leukosis could not be transmitted from chickens to birds of other species. Most virus diseases affect closely related species. The best known virus diseases of chickens, chicken pox and fowl pest, affect closely related species. Leukosis, the cause of which passes filters, apparently is different from the well known virus diseases.

M7. On Filterable Viruses of Leukosis and Sarcoma of Chickens. J.

FURTH, Cornell University Medical College, New York, N. Y.

Avian sarcoma and avian leukosis are caused by numerous filterable agents. These agents are characterized by the histological appearances of the alterations they produce. It is significant that all transmissible

tumors and leukosis of chickens that have been passed through several successive passages and have been thoroughly investigated have been found to be caused by filterable agents and that no two of these agents are identical. The agents of sarcoma stimulate only one type of mesenchymal cell to apparently neoplastic growth, while the agents of leukosis usually stimulate several closely related types of cells. The agent of our leukosis, strain 1, produces erythroleukosis and myeloblastic leukemia (Ellermann); the agent of strain 2 produces hemocytoblastosis, myelocytomatosis, and endothelioma; and the agent of strain 5 produces neurolymphomatosis (Pappenheimer, Dunn and Cone).

Avian neoplasms differ from those of mammals in that the neoplastic cells are carriers of the virus, and there is no evidence that unrestricted multiplication of avian tumor cells may occur in the absence of such a virus. Until recently search was made for new types of viruses of avian tumor and leukosis; now the existence of avian neoplasms transmissible only by viable cells requires investigation.

Sarcoma may be transmitted to chickens immune to leukosis. Furthermore, sarcoma readily produces progressively growing tumors in the injected muscle, whereas the agent of leukosis either perishes after intramuscular injection, or produces the systemic disease or, occasionally, produces mild infiltrations at the site of injection. These characteristics served to isolate the sarcoma viruses that contaminated our transmissible strains (Furth and Stubbs). We find no evidence that the agent of leukosis may mutate into an agent of sarcoma as suggested by Oberling and Guerin.

The data available are insufficient to determine whether these agents are common living entities, as suggested by some investigators, or non-living enzyme like substances, as suggested by others.

M8. Cloacal Infection as a Means of Immunization Against Infectious Laryngotracheitis of Fowls. F. R. BEAUDETTE, New Jersey Agricultural Experiment Station, New Brunswick.

Virus collected from artificially infected birds and preserved by drying is used to produce a harmless infection of the cloacal mucosa. The local reaction disappears in about a week and immunity to respiratory infection is fully developed by the ninth day or earlier. Immunity to artificial infection is known to persist for 9 months and birds have been exposed for 21 months on infected premises without taking the disease. The loss incident to cloacal infection takes place within the first 3 weeks and is less than 2 per cent. Survival of the virus in the cloacal has not

been demonstrated. During the past summer cloacal inoculations were made on 23,800 birds, under commercial conditions.

M10. Studies on the Specificity of the Inactivation of Poliomyelitis Virus by Serum. CLAUS W. JUNGBLUT, College of Physicians and Surgeons, Columbia University, New York.

The obscure and disputed nature of the inactivation of poliomyelitis virus by normal human serum prompted an investigation into the specific character of the neutralization phenomenon as brought about by contact *in vitro* between virus and serum. A number of antiviral, antibacterial and antitoxic immune sera from either the rabbit or the horse were studied for their capacity to inactivate the virus of poliomyelitis (Aycock passage strain) *in vitro*. The technique consisted of injecting intracerebrally into monkeys mixtures of 0.2 cc. of 10 per cent virus suspension and 0.8 cc. of the various antisera after preliminary incubation of 1½ hours at 37°C. and keeping in the icebox overnight. The sera contained no preservative and were not concentrated. Controls on the virulence of the virus accompanied each experiment.

The virus of poliomyelitis, when tested against antisera produced by immunization with other neurotropic viruses, such as herpes, rabies and neurovaccinia, exhibited strict immunological specificity in that no cross neutralization occurred.

Likewise, poliomyelitis virus was not inactivated *in vitro* by agglutinating anti-diphtheria, anti-pneumococcus and anti-meningococcus sera. The same was true when the virus was brought into contact with typical Forssman antisera, such as are obtained by immunization with either guinea pig kidney cells or sheep red cells.

Among the antitoxic type of immune sera, botulinus, tetanus and dysentery antitoxic sera failed to inactivate the virus *in vitro*. Inactivation occurred, however, with 3 out of 6 samples of antitoxic diphtheria serum and with one sample each of antitoxic scarlet streptococcus serum and anti-crotalus serum. While the neutralizing sera were potent in most cases in low dilutions only, inactivation occurred in two instances with serum dilutions up to 1:10. Further experiments demonstrated that 4 of 15 sera obtained from rhesus monkeys after immunization with T. A. or toxoid, had acquired virucidal properties which were not demonstrable before the beginning of immunization. These 4 monkeys when infected intracerebrally with a potent dose of virus either survived without symptoms or developed only an abortive attack of poliomyelitis.

Finally, the following results were obtained with the serum of one Nicaraguan Cebus monkey before and after bilateral adrenalectomy. The normal serum of this animal, in harmony with previous findings, possessed natural virucidal substances. However, when the animal was bled again after the operation, the serum failed to inactivate the virus. The neutralizing property could then be restored by the addition of a small amount of cortical hormone but not of adrenalin to the deficient serum *in vitro*.

The experiments reported above furnish additional indirect evidence that the poliocidal substance in normal human serum may not be a typical antibody in the immunological sense of the word.

M11. A Change in the Contagious Character of a Strain of Swine Influenza. RICHARD E. SHOFÉ, Rockefeller Institute for Medical Research, Princeton, N. J.

Earlier experiments have shown that a filtrable virus and the organism, *H. influenzae suis*, are etiologically essential to the production of influenza in swine. All strains of the disease studied have proven to be highly contagious, both virus and organism transferring from sick to normal animals by contact.

One strain of the disease, under study for 3 years, has been observed to change from a condition of full contagiousness to one in which the disease induced by contact is of an extremely mild character. The clinical and pathological picture presented by swine suffering from the mild illness acquired by contact is characteristic of the disease induced in swine by intranasal infection with the swine influenza virus alone (previously designated as "filtrate disease"). Furthermore, the mild contact-disease is of itself contagious, and it confers a solid immunity to swine influenza. Bacteriological examination reveals that the respiratory tracts of animals infected by contact are free of *H. influenzae suis*. The conclusion reached is that the change in the strain of swine influenza under discussion is one in which the virus alone transfers from sick to normal animals by contact.

In an effort to determine whether the failure of *H. influenzae suis* to transfer by contact was due to some change in the organism itself or whether it was due to an alteration in the associated virus, a fresh and fully contagious strain of swine influenza was obtained from the field. Substitution of the individual etiological components of the fully con-

tagious field strain of the disease for corresponding components of the old stock strain revealed that the change in the contagious character of the latter was due to some biological alteration in the bacterial component and that the virus was in no way responsible.

During the course of the investigation it was observed that swine artificially converted into carriers of *H. influenzae suis* acquired swine influenza instead of the mild filtrate disease following exposure to animals infected with the old stock strain of swine influenza.

M12. Symptomatic Herpetic Manifestations Following Artificially Induced Fevers. R. A. BOAK, C. M. CARPENTER, AND S. L. WARREN, University of Rochester School of Medicine and Dentistry and Strong Memorial Hospital, Rochester, N. Y.

The most frequent complication observed in our clinic in patients subjected to artificially induced fever has been symptomatic herpes. From January 1, 1933 to November 1, 1933 it has appeared in 122 of 200 patients given a total of 352 therapeutic fevers. Typical herpetic lesions developed in from 36 to 72 hours after a fever of from 40° to 41.5°C. The skin lesions were confined to the distribution of the fifth cranial nerve, occurring most frequently in the perioral and perinasal regions. The incidence of the herpetic infection varied during the year, being more frequent and more severe in the fall and winter months. There was a tendency for the infection to occur in a group of patients consecutively treated, while a similarly treated group might not show herpes during the succeeding 2 or 3 weeks. Patients usually showed some immunity following the herpetic infection. In a number of patients herpes did not appear after the first or second fever, but occurred following a third or fourth treatment. Neither the physical method of producing the fever nor the disease for which the patient was treated had any apparent effect on the incidence.

A fatal encephalitis was produced in rabbits with filtrates, bacteriologically sterile, from vesicles of 5 patients, indicating the exhibition of a filtrable virus similar to that of herpes. Successful rabbit passages were accomplished. Rabbits were immunized to the strains of virus recovered from the patients, and these rabbits survived a subsequent intracerebral injection with a known (Frank) strain of herpes which invariably killed the controls. The thermal death time of the Frank strain of virus in a brain suspension was determined at 41.5°C. to be 80 hours.

M13. Active Immunization against Poliomyelitis on Monkeys. MAURICE BRODIE, Department of Bacteriology, New York University and Bellevue Hospital Medical School, New York, N. Y.

In previous reports it has been shown that considerable active immunity can be obtained in macacus monkeys by a single sub-infective dose of virus given intracutaneously and that following the administration of $\frac{1}{2}$ to $\frac{3}{4}$ of a skin infective dose of antigen, immunity developed between the sixth and tenth day and reached its height by the twentieth day. Further experiments have shown the lag period to be about 6 days and the immunity still evident at the end of a year.

Experiments were then conducted to determine: the immunity obtained with an intracutaneous dose of virus, whether a second dose of antigen had any effect upon the immunizing power of the first, and the interval between the two inoculations which would give the maximum response. Each of 4 animals whose serum was shown in the control period to have no neutralizing power for the virus of poliomyelitis received approximately $\frac{3}{4}$ of a skin infective dose of virus and each was given a second inoculation either 6, 10, 14, or 20 days later. Serums collected at various intervals were tested for antiviral substance with the following results: (1) A second dose of antigen did not depress the immunological response to the first. (2) The lag period is shorter and the rate of production of immunity more rapid after a second dose of antigen. (3) When a second inoculation is given during the refractory period of the first, it does not produce additional immunity; given during the rise or height of antibody response to the first, additional protection follows, especially if given during the rise.

The immunity obtained in the above series of animals was then compared with that of 7 convalescent monkeys. Two of these had recovered from a mild attack of poliomyelitis, consisting of a rise in temperature, cerebro-spinal pleocytosis, symptoms, but no residual paralysis. The others had had an attack with widespread paralysis and showed considerable residual deformity. The serums of the monkeys, which had received 2 inoculations at 10- to 20-day intervals, had even better neutralizing power than the serums of the animals which had widespread paralysis.

The tissue immunity of the above and other immunized and convalescent animals was compared with the humoral immunity by measuring the resistance of the animals to intracerebral inoculation and the neutralizing power of the serums. The humoral immunity was decidedly more evident, for although the serums of some animals neutralized as many

as 75 minimal, completely paralyzing doses of virus, they withstood only 3 to 5 doses given intracerebrally. Moreover, the animal whose serum neutralized several infective doses succumbed to a single MCP dose given intracerebrally.

M14. Immunity to Poliomyelitis. Active Immunization. SIDNEY D. KRAMER AND M. SCHAEFFER, Long Island College of Medicine and New York City Department of Health.

A method is discussed for producing an immune response in *Macacus rhesus* with neutralized mixtures of serum and virus.

M15. Louping Ill in Man. THOMAS M. RIVERS AND F. F. SCHWENTKER, Hospital of The Rockefeller Institute for Medical Research, New York, N. Y.

Of 63 individuals, 17 might have had contact with the virus of louping ill (a natural disease of sheep in Scotland). Of the 17, 7 had been in close contact with the inciting agent. Of the 7, 5 possess sera that definitely neutralize the virus. Of the 5, 3 had had encephalitis, one had been sick with an influenza-like disease, and one had not been consciously ill after initiation of work with the virus. Of the 46 people without histories of contact with the virus, only one possesses serum with demonstrable neutralizing properties. The results of our work cannot be taken as proof that man is susceptible to louping ill, but they are sufficiently suggestive to warrant the recording of them in order that workers handling the virus may take precautions to protect themselves. Our findings do indicate, however, that individuals who come in close contact with the virus of louping ill may develop in their sera neutralizing antibodies against the active agent.

M16. Precipitation Reactions of Meningococcus Strains with Immune Serum in Agar Plates in Relation to Antigenic Activity. SOPHIA M. COHEN, Division of Laboratories and Research, New York State Department of Health, Albany

Further data on the serological and immunological properties of stock as compared with recently isolated meningococcus strains have been obtained from a study of their precipitation reactions with immune serum in agar plates. Marked variations were noted in the reactions which occurred with strains of different serological groups. In general, very little precipitation was obtained with strains of groups II and "X" which included several of group IV. In contrast, strains of groups I

and III, irrespective of the length of time since isolation, gave rise to well marked "haloes" on agar plates which contained monovalent group-I or -III rabbit serum or polyvalent horse serum.

Marked variations were also observed in the reactions of different stock strains of groups I and III. Strains of these groups, maintained in this laboratory on dextrose serum agar, which have been used for more than 15 years in the production of therapeutic serum, compared favorably with more recently isolated strains in precipitation reactions, and produced both in rabbits and in horses sera of precipitative activity. Two other stock strains maintained elsewhere were definitely inferior in these properties.

The type of medium in which strains of groups I and III are maintained seems to affect the production of the specific precipitating substance.

M17. A Report of Experimental Immunization Against Proteus hydrophilus, the Etiological Agent in "Red-Leg" Disease of Frogs.

WALTER L. KULP AND DAVID LACKMAN, Connecticut State College, Storrs.

This is a progress report of research in the study of *Proteus hydrophilus*, the etiological agent of "red-leg" disease of frogs. This species is also pathogenic for mice. Immunization of frogs and mice against this organism has been secured by vaccination of those animals with living non-pathogenic species of the *Proteus* group.

M18. Shigella gintotense (Castellani): Its Occurrence in Cultures from Various Sources. EDNA G. JACKSON, Department of Laboratories, Henry Ford Hospital, Detroit, Mich.

The organism has been isolated from the following sources: (1) blood culture in the case of a child admitted to the hospital with an illness running a course not unlike that of paratyphoid fever, (2) urine cultures from five different cases of infection of the urinary tract, (3) bile cultures from three cases, and (4) stool cultures from two cases clinically resembling bacillary dysentery.

The cultural characteristics of the organism are discussed, also the possible significance of its presence in the above cases.

M19. Attempts to Demonstrate a Specific Toxin in Salmonella aertrycke (Var. meleagridis). RUTH CAMERON AND LEO F. RETTGER, Yale University, New Haven, Conn.

While the major effort was devoted to a search for a specific toxin of *S. aertrycke* (var. *meleagridis*), the etiological agent of a paratyphoid epizootic of turkeys, *S. pullorum* and *E. coli* also received some attention.

Filtrates of 12-day old broth cultures of *S. aertrycke* (var. *meleagridis*) were found to be non-toxic for chicks and turkeys, but highly toxic for mice and rabbits. The toxic substances were "non-specific" in nature, since similar effects could be produced with the broth culture filtrates of *E. coli*.

The broth culture filtrates of *S. aertrycke* (var. *meleagridis*) and *E. coli* possessed similar properties. The toxins of both were heat-stable and were destroyed by free hydroxyl ions, but not by hydrogen ions. They were digested with pepsin and trypsin, and could be concentrated by precipitation with ammonium sulphate. The appearance of the "non-specific" soluble toxin in the medium occurred at a time when the active growth phase of the culture had long passed, and after considerable aqueous extraction or autolysis had taken place.

Attempts to demonstrate a specific toxin in filtrates of the turkey organism and of *S. pullorum*, by controlling various environmental factors, resulted in repeated failure. The presence of 10 per cent carbon dioxide in the gaseous environment of growing cultures controlled the hydrogen-ion concentration of the culture medium and stimulated cell growth. The increase in the number of cells resulted in an increase in the potency of the "non-specific" toxin.

By continued grinding of the washed cells of *S. aertrycke*, *S. pullorum* and *E. coli*, a water-soluble substance was obtained which readily killed turkeys and chicks. Alternate freezing and thawing failed to liberate demonstrable toxin (for fowl). A combination of freezing and thawing and grinding did not increase the potency of the filterable toxic substance to any appreciable degree, over what long grinding alone accomplished.

M20. Bacterial Invasions of the Blood Stream in Urology. JUSTINA H. HILL AND LEAH R. SEIDMAN, Brady Urological Institute, The Johns Hopkins Hospital, Baltimore, Md.

By routinely culturing the blood whenever a patient's temperature rises to 101 degrees, a series of over 250 cases showing one or more positive blood cultures has been obtained. The cases are analyzed from the point of view of the relation of the blood stream invasion to the genito-urinary pathology. The bacteria obtained are analyzed in regard to the genera found, their relative incidence, and their relation to organisms present in the genito-urinary tract. Special emphasis is

placed on the significance of *Aerobacter* and of *Proteus* in the genito-urinary tract and in the blood.

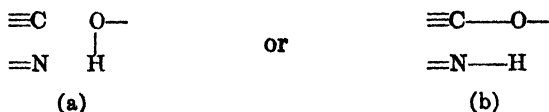
M21. A New Approach to the Chemistry of Immunity. A. E. STEARN, University of Missouri, Columbia.

In spite of the large amount of valuable empirical knowledge on immunity, little, if anything, is really known of immune reactions. It is, therefore, worth while to search for specific *possible* mechanisms, even if only for a starting point toward a new experimental approach and a new analysis of known facts. There are two ways of going about this. One is to look for analogies in behavior with simple systems where mechanism is understood. Thus the Danysz phenomenon has an illustrative analogy in the precipitation of ferric hydroxide by ammonia, wherein less ammonia is required if it be added slowly than if added rapidly.

The second method is to postulate as specifically as possible a mechanism, perhaps guided by the above mentioned type of analogy, consistent with confirmed facts, and to investigate the kind of conclusions such a postulate would yield.

Of the general facts which must be borne in mind, three stand out. (1) General specificity considerations have long been recognized as indicating the importance of steric arrangements. (2) Data from studies such as those of Landsteiner of Obermayer and Pick and of others indicate the importance of chemical groups in a type of bonding. (3) Reversibility considerations indicate that the bonding is not of the ordinary homopolar chemical type.

An attempt has been made to apply quantum mechanical considerations in calculating the potential energies of arbitrarily chosen configurations of certain groups. As an example of the application of the method the group



has been as exhaustively worked out as possible in the present state of the theory. This group is capable of yielding much information. Thus, for example, it can tell something about the hydrolysis of a peptid linkage, and therefore, ostensibly, about proteolysis at least in some aspects (configuration a). It can furnish presumptive information

about the behavior of certain dyes with proteins, or under certain conditions with carbohydrates (configuration b).

It is actually found that under particular ionic conditions a configuration such as (b) can exist with all four atoms close together possessing an energy slightly less than with the C—O and the N—H far apart. The configuration with the smaller energy is the more stable and thus we have a sort of bonding between the two atom pairs without a disruption of the primary chemical valencies; and it is thought that immune bonding may be of this nature. This does not signify by any means that the above atomic groups are even the most important in immune bonds. It does, however, give a definite mode of attack which promises to yield interesting information, and many analogous groups should be investigated in the same way.

M22. The Relation of the Electrical Charge of Bacteria to Their Stability.

HAROLD A. ABRAMSON, Columbia University, New York, N. Y.

Contrary to frequent statements, the electrical charge of bacteria is not decreased when the electrokinetic potential is reduced by the addition of salts not reversing the sign of charge. As the potential decreases and the organisms begin to agglutinate, the net charge of the cells shows, if anything, an increase. It is proposed that the charge as well as the potential must be considered in any attempt to determine the surface chemical factors involved in the regulation of stability. This has hitherto been neglected.

M23. The Chemo-Immunological Properties of the Specific Capsular Polysaccharide of Pneumococcus Type I.

OSWALD T. AVERY,
AND WALTHER F. GOEBEL, Hospital of The Rockefeller Institute for Medical Research, New York, N. Y.

The soluble specific substance of pneumococcus Type I has been chemically isolated both from the bacterial cells and from filtrates of autolysed cultures as an acetyl polysaccharide. So far as could be demonstrated by the methods employed the acetyl polysaccharide absorbs from Type I pneumococcus serum all type-specific precipitins, agglutinins, and protective antibodies. Mice injected intraperitoneally with minute quantities of the acetyl polysaccharide develop a type-specific active immunity to subsequent infection with pneumococcus Type I. In several instances purpura has been observed in mice following the injection of larger amounts of the acetyl polysaccharide. No type-specific agglutinins, precipitins, or protective antibodies were

demonstrable in the serum of rabbits following repeated intravenous injections of the Type I acetyl polysaccharide. The acetyl polysaccharide is readily converted into its deacetylated derivative by treatment with dilute alkali. The chemical and immunological properties of the deacetylated polysaccharide are identical with those of the soluble specific substance in the chemical form in which it was originally isolated. The deacetylated form of the specific carbohydrate is non-antigenic, does not produce purpura in mice, and only incompletely absorbs the type-specific antibodies from Type I antipneumococcus serum. Immunologically, the deacetylated polysaccharide functions solely as a haptén.

M24. Distribution of the Immunizing Antigen in the Pneumococcus.

LLOYD D. FELTON, Harvard Medical School, Boston, Mass.

Pneumococci, both Type I and Type II, were grown in the usual meat infusion broth, collected in a Sharples centrifuge, suspended over night in acetone at room temperature, and finally dried in a vacuum desiccator. By various methods it was found possible to separate the cell constituents into fairly well defined fractions. The first fractionation was a division into acid soluble and acid insoluble. Only about 10 per cent of the immunizing activity of the cell remained in the acid insoluble fraction. This immunity in white mice was largely heterologous in type. The acid soluble fraction, on the other hand, contained most of the immunizing substances; and when the acid fraction was injected into white mice a type specific immunity resulted. However, separation of this acid soluble material into alcohol soluble and alcohol insoluble fractions, gave two fractions varying in immunizing activity. The acid alcohol insoluble fraction produced a type specific immunity in a concentration of 10^{-8} gram (white mice). Conversely, the acid alcohol soluble fraction was less active and produced as well a certain degree of heterologous immunity. The first fraction, acid alcohol insoluble, contained most of the SSS (soluble specific substance) of the cell, the second a type specific and also a species specific substance, the C substance of Tillelt. Whereas the usual vaccines produce a definitely type specific immunity in human beings, these fractions, especially the acid fraction produced active immunity which was largely heterologous in type.

M25. Quantitative Studies on the Precipitin Reaction. MICHAEL HEIDELBERGER AND FORREST E. KENDALL, Columbia University, and the Presbyterian Hospital, New York, N. Y.

A discussion is given of quantitative differences between the reactivities of precipitins produced by the horse and the rabbit. The inhibition zone is explored and a method given for the determination of excess antigen in this zone. The chemistry of the precipitin reaction is discussed and a simple mechanism, capable of quantitative formulation, is given for certain cases of cross-precipitation.

M26. The Prozone Phenomenon in Specific Bacterial Agglutination.

F. S. JONES AND MARION ORCUTT, The Rockefeller Institute for Medical Research, Princeton, N. J.

Two sera with agglutination inhibiting properties and a high titered agglutinating serum from cows naturally infected with *B. abortus* were employed in the observations. The results of experiments indicate that when proper proportions of either are added to a strong *B. abortus* agglutinin, agglutination is inhibited or a prozone developed. Bacteria not agglutinated in the prozone can be centrifuged and resuspended in the same mixture and remain in suspension. When the original supernatant is replaced with salt solution agglutination usually occurs promptly, although where the concentration of prozone serum is considerable an additional washing with sodium chloride may be required to induce clumping.

In respect to electrical charge and migration in an electrical field and increases in antigenic volume, *B. abortus* behaves the same in both inhibitory and agglutinating sera. Differences in behavior between antigens sensitized to the 2 types of sera were brought out in the rate of sedimentation as the result of centrifugation and in the force required to separate films of *B. abortus* under both conditions.

The failure to agglutinate is attributed to the deposition of a substance on the surface of the deposited globulin film which interferes with the cohesive properties of specifically sensitized organisms.

M27. The Influence of the Molecular Weight of Antigen on the Proportion of Antibody to Antigen in Precipitates. SANFORD B.

HOOVER AND WILLIAM C. BOYD, Department of Immunology, Evans Memorial, Boston, Mass.

The ratio, antibody : antigen, in precipitates formed at the equivalence point differs widely in different systems. To account for these differences the authors assume that at the equivalence point the antigen molecule is just completely covered by molecules of antibody. A formula has been derived which permits a calculation of the weight of antibody-globulin thus covering the surface of an antigen molecule of any

weight. The computed and observed ratios have been compared in systems involving hemocyanin (M.W. 2,000,000), the S III hapten (M.W. about 4000), and proteins of intermediate weight—ovalbumin, hemoglobin, and pseudoglobulin. The agreement is very good except in the case of pseudoglobulin; this single discrepancy is probably due to the difficulty of isolating this protein.

M28. Comparative Antigenic Studies on Egg Albumin Denatured by Intense Audible Sound and by Other Means. EARL W. FLOSDORF AND LESLIE A. CHAMBERS, School of Medicine, Univ. of Pennsylvania, Philadelphia.

During the investigation of the bactericidal action of intense audible sound it was observed that a variety of chemical changes occurred including that of rapid coagulation at 25°C. of well-dialyzed solutions of three times recrystallized egg-albumin.

Antigenic studies with the washed coagulum from treated albumin indicate an altered specificity similar to that produced by heating in an alkaline or acid solution. The uncoagulated fraction retains the original specificity. A confirmation of the studies on denaturation of proteins by Wu, Tenbroeck and Li was observed insofar as it was necessary to reproduce them in this study.

There is preliminary indication that the degree of antigenic activity in producing immunity is increased as a result of brief sound treatment. Long treatment redisperses the coagulated fraction into a very opalescent solution which cannot be separated by centrifuging at ordinary speeds, but this mixture yields a precipitate both with anti-native-albumin and with anti-heat-denatured-albumin rabbit sera.

Immunization was carried out in rabbits by the subcutaneous route. The animals were tested for degree of immunization both by intradermal testing and precipitation with the serum obtained after bleeding. Excellent agreement both with respect to degree of immunization and to antigenic specificity was observed in the results of the Arthus phenomenon, in the intradermal testing, and in serum precipitation.

M29. Disimmunization and Accompanying Phenomena. REUBEN L. KAHN, University of Michigan, Ann Arbor.

Disimmunity is a term chosen to designate the reversal of the immune state. The prefix "dis" is used in the same sense as in "disarm." Disimmunity is not synonymous with non-immunity or susceptibility since the latter terms do not assume previous immunity. Under con-

ditions of infection, the disimmune state implies that the parasite has gained the upper hand over the host following the immune state when the host had the upper hand; in the former state, there is generally widespread invasion of the parasite throughout the body in contradistinction to the immune state when the parasite is kept localized. Under experimental conditions, an animal, such as a rabbit, that is immunized to some bacterial protein can be disimmunized if it is "overwhelmed" by the intravenous injection of a relatively large amount of antigen. During the disimmunized state, the capacity of the skin to react to injected antigen, disappears. With regard to the capacity of the serum to react with antigen *in vitro*, precipitins in a protein-disimmunized animal disappear, while agglutinins in a bacterial-disimmunized animal do not appear to be affected. The term "desensitization" has heretofore been applied to the condition wherein the capacity of the skin to react with antigen disappears in a protein-immunized animal following an intravenous injection of the antigen. It is believed that the term disimmunization is more applicable to this condition than desensitization. The latter term brings to mind tissue hypersensitiveness and anaphylaxis and is furthermore assumed in clinical literature to be equivalent to a return to normality by patients suffering from hay fever or allied conditions.

The disimmunized state resulting from the intravenous injection of relatively large quantities of antigen in rabbits is of short duration, lasting from some hours to a few days. If a horse serum-immunized animal is injected intravenously with, let us say, 1.0 cc. of serum per kilogram of body weight, the skin loses its capacity to react with antigen and the precipitins disappear within an hour after the injection. Soon the animal reverts to the immunized state. Daily repeated disimmunizing (intravenous) injections do not appear to prolong the disimmunized state. In the case of specific disimmunizing injections of bacterial-immunized rabbits, the disimmunizing effect seems to be limited to the tissue (skin) reactions, the serum agglutinins showing no change. With regard to the phenomena that accompany the disimmunized state, inflammatory skin lesions resulting from the injections of suspensions of specific organisms in bacterial-immunized rabbits change from red to purplish black soon after an intravenous disimmunizing injection. This change in the appearance of the skin lesions was noted if the disimmunizing injection was given up to 4 days after the skin injection. It was also observed that areas in the skin wherein quantities of specific bacterial suspension had been injected insufficient

to produce an inflammatory response, became purplish-black soon after a disimmunizing injection. The organisms employed were *B. typhosus*, *B. paratyphosus* A, *B. coli*, and *Streptococcus hemolyticus*.

M30. Serum Reactions in the Disimmunized State. REUBEN L. KAHN AND ELIZABETH B. McDERMOTT, University of Michigan, Ann Arbor.

It was indicated in the preceding discussion that an intravenous disimmunizing injection of specific antigen in a protein-immunized rabbit causes the disappearance of the serum precipitins, while a similar specific injection in a bacterial-immunized rabbit apparently does not affect the serum agglutinins. This difference in the behavior of agglutinins and precipitins during the disimmunized state raised the question as to the relation between these antibodies, since it is widely assumed that they are but different manifestations of a single type of antibody. To throw light on this question, complement fixation tests were made on the serum of protein-disimmunized and bacterial-disimmunized rabbits. It was found that the complement fixation reactions were negative in the case of the protein-disimmunized animals similar to the precipitin reactions, and positive in the case of the bacterial-disimmunized animals similar to the agglutinin reactions. This finding would indicate that it is not necessary to assume that the precipitins and agglutinins represent two basically different types of antibodies. If this were so it would also be necessary to assume that complement fixation reactions with protein solutions are basically different from complement fixation reactions with bacterial suspensions. It is likely that the difference in the agglutinin and precipitin reactions in the disimmunized state is largely due to the difference between the total reacting surface of the minute colloidal particles of the protein antigen and the comparatively large particles of the bacterial antigen. The former colloidal particles might readily adsorb large quantities of precipitins while the bacterial particles might adsorb only comparatively small quantities of agglutinins.

M31. Correlation of Agglutinative Types and Endotoxin. CAROLINE R. GURLEY, MARGARET CASTELDA AND RUTH GOLDBERG, New York City Department of Health.

The object was to learn more concerning the width of protection in terms of absorption groups, as a basis for increased effectiveness of therapeutic sera against endotoxic strains and concerning the degree of correspondence between results of tests in mice and in rabbit skins.

Classification by absorption: Comment is made on limitations of method and need of quantitative tests. Strains studied were chiefly from erysipelas and the blood or chest-fluid in pneumonia, usually pneumococcus-induced. A moderate proportion from pneumonia proved of erysipelas agglutinative types, of which we have at least 3, probably 4, and unclassified strains.

Results: (1) The correlation between agglutinative types and endotoxic power is marked. This is especially exemplified in Erys. Type II; (2) The need is stressed of a serum against Sub *b* of that type whose virulence and wide distribution has been shown (from septicaemias complicating pneumonia, from puerperal sepsis and cellulitis); (3) strains related to certain erysipelas ones, by absorption, are in the throat in some severe cases of angina, 2 of which were sent us as *S. epidemicus*, being considered responsible, respectively, for the epidemics of septic sore throat in Boston (1911) and Chicago (1912); (4) the rabbit-skin test does not compare well with the mouse test but is valuable pending raising of virulence or with strains non-virulent for mice.

M32. The Antigenic Value of Unprecipitated and of Alum Precipitated Tetanus Toxoid. D. H. BERGEY AND S. ETRIS, Department of Research in Biology, The National Drug Co., Philadelphia.

We have immunized several groups of guinea pigs with 3 doses of 1 cc. each using different lots of unprecipitated tetanus toxoid. The toxins, from which the toxoids were prepared, contained from 3000 to 10,000 M.L.D. The antigenic response to immunization with the toxoids was not fully comparable to the toxicity of the original toxins as measured by the determination of the antitoxic content of the serums, nor as measured by the amount of standard toxin required to kill the immunized animals.

We have also immunized several groups of guinea pigs with a single dose of 1 cc. of alum precipitated toxoid. The antitoxic content of the serums in these guinea pigs was apparently higher than that in guinea pigs receiving 3 doses of unprecipitated toxoid of the same degree of toxicity of the original toxin, indicating that the immunity response from a single dose of alum precipitated toxoid is equal to or greater than the following the use of three doses of unprecipitated toxoid.

We have administered a single dose of alum precipitated toxoid to 34 human beings. Preliminary tests of the serums (25 days after the injection of the toxoid) showed that 5 young adults had developed

from a trace up to 10^3 of a unit of antitoxin. Subsequent tests (3 months after treatment) are believed to show a much higher antitoxic content. The results of these later tests will be discussed in the paper.

M33. The Antigenic Relation of Diphtheria Organisms of the Gravis and Mitis Strains. S. ETRIS, Department of Research in Biology, National Drug Company, Philadelphia.

Two forms of diphtheria bacillus have been reported by Anderson, Happold, McLeod and Thompson: Gravis, associated with severe toxic cases of diphtheria, giving poor response to antitoxin treatment, and Mitis, with milder cases responding to antitoxin.

Gravis strains were received from Doctor Ewing, Oxford University. These strains had been isolated in Berlin, Leeds, London, and another Gravis strain was obtained from New York. Toxins were prepared from each strain and the M.L.D. and L+ dose estimated. Formaldehyde (0.4 per cent) was added and the mixture incubated until a 5-cc. dose produced no local or general reaction in guinea pigs. After detoxication was complete each lot was precipitated separately by the addition of alum, washed with salt solution, and diluted so that each cubic centimeter of the toxoids contained an equal number of original M.L.D.

Guinea pigs and rabbits were injected with a series of subcutaneous doses and after a resting period were bled from the heart.

The present studies were undertaken with the idea of determining: (a) The extent of neutralization of the Gravis toxin by standard antitoxin. (b) The antitoxic response in guinea pigs and rabbits injected with alum precipitated Gravis toxoid. (c) A comparative study of the unit value of antitoxic serum from guinea pigs treated with alum toxoid of the Gravis strains and that of the Park 8 strain. (d) The ability of normal guinea pigs to withstand a lethal dose of diphtheria bacilli after injection of Gravis guinea serum, using standard antitoxin as control.

The tests were controlled using an emulsion of the organism of the Park 8 strain and alum toxoid, prepared from the Park 8 strain.

The results of the studies are discussed.

M34 A Comparative Study of Freshly Isolated and Stock Strains of B. pertussis in Relation to Their Antigenic Properties. JULIA M. COFFEY, New York State Department of Health, Albany.

A comparative study of recently isolated and old stock strains of

B. pertussis was made in order to determine a basis for the choice of strains to be used in the production of vaccine for the prevention and treatment of whooping cough. Recently isolated cultures differed from stock strains in morphology, cultural characteristics, pathogenicity, and the intracutaneous reactions induced in rabbits, but no marked differences in their antigenic value were demonstrable by the agglutination titers of sera of immunized rabbits, or by the intracutaneous reactions induced in rabbits before and after immunization.

M35. A Study of B. pertussis Cultures by Means of Animal Inoculation.

PEARL KENDRICK AND GRACE ELDERING, Michigan Department of Health, Western Mich. Division, Grand Rapids.

The experiments reported are part of a study to determine the effects of the parenteral injection of cultures of *B. pertussis* into laboratory animals—particularly mice and guinea pigs. Using a recently isolated culture, the intraperitoneal dose which appeared to be always fatal for an animal of a given weight was determined and used as a measure in studying different culture suspensions. Agglutination tests were performed on the suspensions used for animal tests, in order to determine whether there were any correlation between the serological reaction of a culture and its pathogenicity for the test animal. The pathogenicity of the cultures was found not to be directly related to the period which had elapsed since their isolation. Marked differences were observed in the killing property of living and killed cultures. A delayed toxic effect of certain killed cultures was observed in guinea pigs. After a period varying from around two weeks to over a month, some of the inoculated pigs showed marked loss of weight, weakness and partial paralysis of the hind quarters and finally death. The possible significance of the various findings with respect to *B. pertussis* vaccines is discussed.

M36. Antigenic Structure of Staphylococcus aureus and Its Variants.

RACHEL E. HOFFSTADT, GUY P. YOUNG AND WESLEY CLARK, University of Washington, Seattle.

The antigenic structure of the undissociated *Staphylococcus aureus*, the rough variant and the G, gonidial form, were studied from the following viewpoints:

1. Agglutination of all strains by monovalent, divalent and trivalent salts in varying concentrations. No monovalent salt agglutinated any form. Bivalent salts of Pb, Cu, and Fe, and all trivalent salts

used agglutinated in some concentration all forms. Twice the concentration of trivalent salts and divalent necessary for the agglutination of the S were needed for the agglutination of R and from 2 to 4 times the concentration for the agglutination of G. High concentrations of Al and Cr retarded agglutination of all forms.

2. Agglutination and absorption of all strains by specific immune sera. Immune sera were prepared with living antigen for the R and G forms and for S by autolysing the organism with CO₂ under pressure. S and R organisms agglutinated the S, R and G sera. The G organisms agglutinated only the G sera. As shown by the absorption tests, S and R organisms contained S, R and G agglutinogens and the G organism contained S and G and no appreciable R agglutinogens.

3. Extraction of the specific carbohydrate present in all forms. A method is described for extraction of the specific soluble substance in the undissociated form and R variant with a report of the yield.

M37. Certain Host Factors Involved in the Protective Action of Anti-pneumococcus Serum in Experimental Infections in Rabbits.

KENNETH GOODNER, Hospital of The Rockefeller Institute for Medical Research, New York, N. Y.

When relatively small amounts of specific antiserum are administered to rabbits as a protective measure against Type I pneumococcus infection some animals die whereas others survive. Among the factors which appear to play a rôle in the determination of the outcome are breed, weight, and white blood cell count.

M38. Utilization of Carbohydrates and Salts of Organic Acids by C. diphtheriae in the Production of Strong Toxin. GEORGE F. LEONARD AND AUGUST HOLM, Biological Laboratories, E. R. Squibb & Sons, New Brunswick.

Metabolism studies were made of *C. diphtheriae* when grown on Kirkbride, Berthelsen and Clark's semi-synthetic infusion-free medium, which was chosen on account of its ability to support the production of a fairly uniform and satisfactory toxin in respect to M.L.D., L+, and Lf values. The composition of this medium is such, that the peptone is the only factor of unknown composition; it is easily prepared and the nitrogen content is low. Various carbohydrates and salts of organic acids in different amounts were added to the infusion-free medium. The effect of these additions in respect to growth and toxin production was investigated. The optimal requirements were found to be the ad-

dition of 0.45 per cent maltose and 0.75 per cent sodium acetate. The regular production of a uniform toxin resulted, having an M.L.D. of 0.001 cc., an L+ of 0.04 cc. and an Lf value of 32 to 34 units per cubic centimeter. The medium was sterilized by heat.

M39. A Strain of Cl. tetanus Isolated from a Human Case of the Disease.

G. D. CUMMINGS, Bureau of Laboratories, Michigan Department of Health, Lansing.

The morphological, cultural, serological, and toxin characteristics of a strain of *Cl. tetanus* isolated from a section of human uterine tissue are presented. The tissue was removed from the uterus of an abortion case when the patient was already clinically considered ill with tetanus. The strain was isolated by the Fildes technique. The anerobic method used was the author's modification of Fildes' method.

The organism measures from 0.3 to 0.5 μ in diameter. The length varies from a few μ to several field diameters. The Gram reaction varies with age of the culture. Flagella staining shows the organisms to be peritrichous. Typical spores are formed. Pronounced motility is observed in the hanging drop.

The organisms grow easily on plain and dextrose veal infusion broth. Gelatine is liquified and hydrogen sulphide is produced. Cooked meat medium is slowly digested while coagulated egg albumin is not attacked. No carbohydrates are fermented. Indol is produced. No hemolysis is indicated on streaked or poured blood agar plates.

The culture agglutinates with type III agglutinating serum.

The strain produces a potent toxin which kills 350-gram guinea pigs with typical tetanus. The toxin can be neutralized with standard tetanus antitoxin.

M40. Biological Characters of the G and M Variants of a Hemolytic Streptococcus. CHRISTOPHER ROOS, JOHN REICHEL AND JANET CLARK, Mulford Biological Laboratories, Sharp & Dohme, Glenolden, Pa.

A virulent culture of hemolytic streptococcus (No. 1685), isolated from erysipelas lesion and cultured in calf brain broth containing 5 per cent normal rabbit blood, dissociated into G and M variants.

The M variant retained all the characters of the original culture, except for a gradual loss of virulence which was regained upon passage through mice.

The serum of a horse receiving the M variant showed passive proteo-

tion for mice injected with the original virulent culture. The boiled suspension of the M variant absorbed completely the protective substance from homologous anti-serum and from a high titre serum obtained from a horse receiving the original virulent culture. Agglutinin and agglutinin absorptive properties were about equal those of the original virulent culture.

The G variant differed markedly from the original culture in colony appearance, but fermented the same carbohydrates. Hemolysis of blood cells in plates was more marked. There was complete loss of virulence, but no loss of toxigenicity. Virulence was not increased upon passage through mice. Agglutinogenic, agglutinative and agglutinin absorptive properties were retained.

There was however a very marked loss of type specific antigen as shown by the absence of protective substance in the serum from a horse receiving the G variant. Boiled suspensions of the G variant showed very weak absorptive property for the protective substance in high titre monovalent serum from a horse receiving the original virulent culture.

The G variant represents the non-virulent phase of hemolytic streptococcus and is deficient in type specific antigen, which accounts for the low protective titre in the serum of the horse receiving the G variant. The M variant is the virulent phase of the organism and possesses the type specific antigen. The serum from the horse receiving the M variant shows type specific protective substance and confers passive protection to white mice against the culture of high virulence.

M41. A Toxic Filtrate Obtained from Hemophilus influenzae. CHRISTOPHER ROOS, JOHN REICHEL AND JONATHAN E. WOOD, Mulford Biological Laboratories, Sharp & Dohme, Glenolden.

Filtrates from a freshly isolated culture of *H. influenzae* were found toxic for white mice and guinea pigs. The M.L.D. for 20-gram mice given intravenously was 0.3 cc. and for young guinea pigs weighing 250 grams was 0.03 cc. injected intracisternally. One M.L.D. caused death in from 16 to 48 hours. The filtrate stored at -10°C . lost about 50 per cent of its toxic property the first month.

The culture was isolated from the spinal fluid of a $2\frac{1}{2}$ year-old child. The toxic filtrates were produced by growing the culture in 0.3 per cent veal infusion agar and 5 per cent heated normal horse blood, in an atmosphere of 25 per cent CO_2 —75 per cent oxygen and filtered through paper after 3-day incubation at 37.5°C .

Several other cultures of *H. influenzae* of recent isolation from like sources have yielded toxic filtrates of less than one-half the M.L.D.

According to agglutinogenic, agglutinative and agglutinin absorptive properties the culture (No. 2099) represents a common type of *H. influenzae* found in spinal fluids.

Seven other strains of *H. influenzae* isolated within the past two years from spinal fluids belong to the same type.

H. influenzae anti-serum no. 77217, from horses, mixed with the toxic filtrates in proportions up to 1:3 and incubated for one hour at 37°C. neutralized the lethal effect for the test animals.

Aside from showing the toxic M.L.D. strength of the filtrate (S.C. no. 2099), it has been shown that anti-serum (no. 77217) neutralized the toxin.

M42. Tuberculosis of Cold-Blooded Animals. JOSEPH D. ARONSON AND HOWARD J. HENDERSON, Henry Phipps Institute, Philadelphia.

In previous publications there was described the occurrence of spontaneous tuberculosis in fish from which was isolated an acid- and alcohol-fast bacterium for which the name *M. marinum* was suggested. Subsequently an acid-fast but not alcohol-fast bacterium was isolated from organs of garter snakes (*Thamnophis sirtalis*), dying from spontaneous tuberculosis, for which the name *M. thamnopheos* was suggested. Recently spontaneous tuberculosis has been noted in two iguanas (*Iguana tuberculata*), a frog, a turtle, and a rattle snake found dead in the Philadelphia Zoological Gardens. From the different organs of these animals acid- and alcohol-fast organisms have been isolated. The cultural characteristics of these organisms differ, and in one instance the cultural characteristics of the organism isolated from the spleen of an iguana were different from the characteristics of an organism isolated from the lungs and liver of the same animal.

The cultures isolated from the frog and from the iguanas differ in some characteristics from *M. marinum*.

Guinea pigs inoculated with various strains of *M. thamnopheos* failed to react to Old Tuberculin prepared from human tubercle bacilli or to tuberculin prepared from the homologous organisms, but in some instances gave an accelerated nodular formation.

Guinea pigs infected with *M. marinum* isolated from fish, and from one of the iguanas reacted to Old Tuberculin.

By means of agglutination and agglutination absorption experiments it was found that the various strains isolated from the garter snakes (*M. thamnopheos*) were agglutinated by their specific anti-serum, but failed to be agglutinated by antisera prepared from cultures isolated from other cold-blooded animals. On the other hand, the cultures iso-

lated from salt water fish (*M. marinum*), the cultures isolated from the frog (*M. ranae*), and from the iguanas (*M. iguana*) showed cross agglutination and absorption.

M43. Experimental Mouse Tuberculosis. ESMOND R. LONG, Henry Phipps Institute, Philadelphia.

Experimental tuberculosis in the mouse, produced by human-type tubercle bacillus infection, proved interesting because of the course of development of the disease and the final notable tendency for once extensive lesions to heal.

Following injection with the relatively large dose of 1.0 mgm., injected intraperitoneally in 25 to 30-gram mice, a period of generalization ensued in which tuberculosis waxed and waned in different organs in a fairly regular sequence. First lesions other than superficial involvement of the peritoneum and the regional lymphoid tissue, occurred in the spleen. Infection of this organ was most extensive in the period from 14 to 50 days after inoculation. After 90 days bacilli were infrequently seen, although in a few cases bacilli were found in considerable number. Maximal involvement of the liver closely followed that of the spleen, occurring in the period from 20 to 50 days after inoculation. Subsequently pathological change waned much as in the spleen. In the lungs well developed lesions first appeared 30 days after inoculation, and thereafter increased in severity, seething with bacilli, up to about 80 days, and then diminished. The majority of tuberculous lesions seen after that period showed considerable evidence of healing. In the kidneys bacilli were first seen in the glomerular capillary blood at 4 days, but without lesions. Between 45 and 100 days lesions, often containing large numbers of bacilli, were present, but inconstantly. After 100 days bacilli were frequently seen, but the scars of healed lesions were frequent.

The healing process was best observed in the lungs. In the period of maximum involvement consolidations up to 2 mm. in diameter were common. The alveoli in these regions were filled with large phagocytic cells often so packed with bacilli that the cytoplasm could not be distinguished. At a later period the areas were much as before in extent, but the bacilli had largely disappeared, the alveoli being filled with degenerated phagocytic cells with abundant vacuolated cytoplasm frequently containing brown pigment. Still later (150 to 180 days after inoculation) the areas were smaller and the cells for the most part small, with little cytoplasm, except for a central zone in which the large vacuolated

cells persisted, with or without bacilli. No appreciable necrosis of lung tissue occurred, and in the latest period studied formerly tuberculous regions were represented by small zones of small monocytes (lymphocytes?) and slightly thickened alveolar walls. Some relation between the process of healing and forced diets of animals was noted, which will be reported subsequently.

M44. The Utilization of the Bordet-Gengou and Löwenstein's Media as a Substitute for Guinea-pig Inoculation in Detecting Tubercle Bacilli in Sputums. LUCY MISHULOW, MARIE ROMANO, MILDRED MELMAN, AND CAMILLE KERESZTURI, N. Y. Department of Health, Research Laboratories, New York.

This study was undertaken as a continuation of a previous investigation in which it was found that tubercle bacilli grow freely and rapidly on the Bordet-Gengou and the Löwenstein's media and that the growth of the human and bovine type is so characteristic on these media that they can readily be differentiated on their colony morphology. The results were so encouraging that it seemed desirable to test these media further with a view of utilizing them in the diagnosis of tuberculous infections and, if the results warranted, to substitute them for guinea-pig inoculation.

With this object in view we examined 234 specimens of sputum from 161 definite and suspected cases of tuberculosis for acid fast bacilli by smear, culture on Bordet-Gengou and Löwenstein's media, and by guinea-pig inoculation. The sputums of these cases were previously found negative on routine smear examination. By using a special technique of making the smears, 46 specimens or 19.7 per cent were found positive on direct examination. The remaining 188 specimens were negative on both direct examination and after concentrating the sputums. In no instance in this series was a positive result obtained on smear examination of the concentrated sputum where it was negative on the direct examination.

The 188 specimens that were negative on all smear examinations were cultured on Bordet-Gengou and Löwenstein's media and also inoculated into guinea pigs. Through a misunderstanding the first 123 specimens were collected into unsterilized cups and as a result a large number of specimens were contaminated, also a large number of the guinea pigs died of other infections. The subsequent 65 specimens were collected in sterilized cups and very few of the specimens were lost through contaminations.

In the first series of 123 specimens the percentage of positives was very small for both the cultures and the guinea pigs—2 positive on culture and 3 positive on guinea-pig inoculation.

In the second series of 65 cases there were 10 positive on culture (11.8 per cent) and 8 on guinea-pig inoculation (9.4 per cent). Where the specimens were collected in sterile containers the results were more favorable on the culture than on guinea-pig inoculation.

Conclusions: Although the number of sputum specimens in this series is not very large, the following conclusions may be drawn: (1) By employing a special technic in making smears of suspected tuberculous sputums the results of the examination of unconcentrated sputums were equal to those of concentrated sputums. (2) From the results of this investigation it seems that culturing sputums on Bordet-Gengou and Löwenstein's media could be substituted for guinea-pig inoculation.

M45. Double-zone Beta Hemolytic Streptococci. J. HOWARD BROWN,
The Johns Hopkins University, Baltimore.

Since 1913 the author has occasionally encountered strains of hemolytic streptococci which, under suitable conditions in blood agar, form colonies surrounded by double zones of hemolysis. The first zone to appear is a small clear beta zone immediately surrounding the deep colony. Surrounding this central zone there later appears, sometimes during the period of incubation, sometimes after the plates have been refrigerated, an outer zone of partial hemolysis. The appearance of the outer zone is usually preceded by the appearance of a ring or border of darkened red corpuscles immediately surrounding the inner clear zone and this ring of darkened corpuscles usually persists after the outer zone has appeared. A similar ring of darkened corpuscles usually surrounds the outer zone. There is no greenish or brownish discoloration of corpuscles such as occurs with streptococci producing alpha zones. Because there is complete hemolysis adjacent to the colony these zones are described as "beta double zones."

The first streptococci found producing zones of this description were from cows' milk. Since 1930 over 20 strains producing beta double zones in blood agar have been isolated from human sources (throat, vagina, cervix, lung, blood, abscess, etc.) and in some cases were undoubtedly of pathogenic significance.

A cultural study has been made of over 30 strains of streptococci of bovine and human origin. All of the strains studied to date have fermented glucose, sucrose and trehalose; none has fermented mannitol, raffinose, inulin or sorbitol. All strains except 4, which were from a

single herd of dairy cows, fermented salicin. Half of the strains fermented lactose. Nearly all of the strains hydrolyzed sodium hippurate. Hemolysis of washed rabbit blood cells by serum broth cultures within two hours at 37°C. could not be correlated with source or pathogenicity of the streptococci; the bovine strains produced slight or no hemolysis, but the human strains produced all degrees of hemolysis or none. Inability to ferment lactose appeared to be closely correlated with virulence for mice. All of the non-lactose fermenting streptococci when injected intraperitoneally killed mice within 24 hours. Only two of the lactose fermenting strains killed mice. All of the bovine strains fermented lactose and none was pathogenic for mice. The final pH produced in glucose broth was usually from 4.4 to 4.6 and did not serve to differentiate human from bovine strains.

The facility with which beta double zones are produced in blood agar is influenced by various factors such as the number of colonies in the plate, the period of incubation, exposure to lower temperatures, the kind of blood used and possibly by other constituents of the media. For the present no explanation of the phenomenon is offered. Strains of *Streptococcus pyogenes*, such as those from scarlet fever, erysipelas and septic sore throat, have not been found to produce double zones and differ in other respects from the strains here described as, for instance, in the reaction with sodium hippurate and in the final hydrogen ion concentration in glucose broth. It does not seem likely that all of the streptococci forming beta double zones belong to a single species, but they form a group which should be differentiated from other beta hemolytic streptococci. Unless the proper technique for recognizing these strains were employed they would be and undoubtedly have been confused with other hemolytic streptococci under the general designation of *Streptococcus hemolyticus*. The author is opposed to the use of the terms *Streptococcus hemolyticus* and *Streptococcus viridans* as names of species.

M46. Studies on Minute Hemolytic Streptococci. I. The Cultural Characteristics of Minute Hemolytic Streptococci. ELEANOR A. BLISS AND PERRIN H. LONG, The Johns Hopkins University Medical School, Baltimore.

In the course of investigations upon the aerobic bacterial flora of the rhinopharynx in normal individuals and in those suffering from upper respiratory tract infections, the occurrence of minute hemolytic streptococci has been observed.

These organisms are isolated from 5 per cent rabbits-blood, neo-pep-

tone agar surface plates, and poured plates. The colonies are practically invisible and the area of hemolysis, while small and slow in developing, is distinctly of the beta type. The organisms are smaller than ordinary beta hemolytic streptococci; they tend to occur in short chains and masses, and are often pleomorphic. These streptococci ferment trehalose and do not hydrolyse sodium hippurate. They have been isolated rarely from normal individuals. The majority of our strains have been recovered from the throats of individuals ill with diseases in which the hemolytic streptococcus is generally considered to be of etiological importance.

M47. A Systematic Study of Microorganisms Which Decompose the Specific Carbohydrates of the Pneumococcus. GRACE M. SICKLES AND MYRTLE SHAW, Division of Laboratories and Research, New York State Department of Health, Albany.

The morphological, biochemical, and cultural characteristics of certain bacterial species which decompose the carbohydrates of the pneumococcus are recorded.

The microorganism which decomposes the carbohydrate of pneumococcus Type I and that which acts on the carbohydrate of Type II are similar in their physiological activities. Both are very pleomorphic. Neither strain grows on ordinary beef-extract peptone agar. However, the action of each on pneumococcus carbohydrate is quite specific.

Four strains from different sources, which split the carbohydrate of pneumococcus Type III, are similar morphologically, resembling ordinary aerobic spore-bearers in form and biochemical properties, and growing readily on standard media.

The microorganism that decomposes the nontype-specific carbohydrate, obtained from a strain of pneumococcus Type I which had become atypical (degraded) in the tissues of a horse, is likewise an organism which grows on standard media. It is a small, non-motile, non-spore-forming rod and, except for its ability to split the pneumococcus carbohydrate, does not appear to differ from the ordinary bacteria of the genus *Flavobacterium*.

M48. The Fermentation of Glycogen by Pneumococci. L. A. BARNES AND BENJAMIN WHITE, Antitoxin and Vaccine Laboratory, Mass. Department of Public Health, Boston.

The purpose of this communication is to report the results of a comparative study of the ability of Types I, II, III, and V pneumococci to

ferment dextrose (Merck), inulin (Merck), mammalian glycogen (Pfanzstiehl), and glycogen prepared from scallops. The cultures used were laboratory stock strains.

Benedict's qualitative tests for reducing sugars made on one per cent solutions of the 4 carbohydrates before and after autoclaving were negative in the cases of inulin and the 2 samples of glycogen, while dextrose gave typical reactions.

One-tenth per cent solutions of the 4 carbohydrates in peptone water were autoclaved, and then 10 per cent normal horse serum added. Each medium was inoculated with 0.2 cc. of a broth culture of Types I, II, III, and V pneumococci, respectively. A portion of each medium was left uninoculated to serve as a control. Hydrogen-ion determinations using a Hellige apparatus after 24 and 96 hours incubation showed pH values in the cultures ranging from 5.5 to 6.9. There was no acid production in the controls.

The results show that the 4 pneumococcus types used ferment glycogen to essentially the same degree as they do dextrose and inulin. This fact tempts one to speculate upon a possible relationship between this utilization of glycogen and toxic symptoms of obscure origin in certain cases of lobar pneumonia.

M49. The Pneumococcidal Powers of Sodium Oleate and Sodium Ricinoleate. L. A. BARNES AND CHARLOTTE M. CLARKE, Antitoxin and Vaccine Laboratory, Mass. Department of Public Health, Boston.

The observation by Larson and Nelson that sodium ricinoleate in small amounts exerts a rapid lethal effect upon pneumococci without impairing their antigenicity has a practical application in the preparation of vaccines used for the routine production of antipneumococci serums. Experiments were therefore conducted to determine the minimum amounts of sodium ricinoleate, and sodium oleate (Merck) necessary to devitalize broth cultures of pneumococci of Types I, II, and III used in this laboratory.

Two per cent stock solutions of the soaps were made in physiological salt solution. Preliminary tests showed that the reagents in a final concentration of 0.01 per cent killed Type I pneumococci immediately.

Three series of dilutions of the 2 soaps were made in which the amounts of the reagents ranged from 0.4 per cent to 0.000000004 per cent. Each series was inoculated with 0.1 cc. of broth cultures of Types I, II, and III pneumococci, respectively. After 24 hours incubation at

37.5°C., microscopic examination and subcultures showed that 0.004 per cent sodium ricinoleate and 0.0004 per cent sodium oleate were effective lethal amounts for all 3 types of pneumococci. Repetition of the experiments yielded identical results.

From these experiments, therefore, it appears justifiable to conclude that sodium ricinoleate, 0.004 per cent and sodium oleate, 0.0004 per cent, are approximately the minimal pneumococcidal concentrations of the soaps. The antigenic effect of cultures killed by such treatment is the subject of a subsequent study.

M50. The Characteristics of a Probable New Member of the Neisserieae.

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A Gram-negative coccus, isolated from lung sputum in two cases, by staining reactions and morphology belongs with the Neisserieae. It resembles the *N. catarrhalis* in fermenting no sugars. Culturally, it more resembles *N. meningitidis* but is more delicate, dying out quickly on artificial media; furthermore, in contradistinction to *N. catarrhalis* it forms perfect emulsions in salt solution. It does not grow at room temperature (21°C.) or anaerobically. Since this organism does not fit with any of the described members of the group, the designation *Neisseria pseudo-catarrhalis*, nov. sp., is proposed.

M51. The Chemistry of the Cellular Constituents of the Genus Brucella.

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Central Brucella Station, Michigan State College, East Lansing.

A method is described by which bacterial cells of the 3 species of the genus *Brucella* were separated into chemically individual fractions. Preliminary findings of the chemical and biological study of these fractions are reported.

The *Brucella* were found to be characterized as a group by the absence of free simple sugars, by the occurrence of nonprecipitating polysaccharides only, by a large proportion of water extractable proteins, and by cell lipids analogous to the conventional types found in higher organisms.

The species of the genus *Brucella* could be differentiated one from another by the relative proportions, rather than kind, of 2 biologically inactive polysaccharide, and 2 lipid constituents. *Br. melitensis* was distinguished further from the other 2 species, by the occurrence of a non-protein, non-polysaccharide, precipitating antigen of a type hitherto undescribed.

THE GROWTH OF *BACILLUS MEGATHERIUM* IN RELATION TO THE OXIDATION-REDUCTION POTENTIAL AND THE OXYGEN CONTENT OF THE MEDIUM

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It is now believed by many bacteriologists that the growth of anaerobic bacteria is controlled by the oxidation-reduction potential of the medium. This seems to have partially supplanted the old belief in the rôle of oxygen. In spite of the attractiveness of the new view, there seems little experimental basis for its adoption, aside from certain observations like those in relation to the ability of anaerobes to grow under atmospheric pressure in the presence of reducing substances. But in ordinary culture media the oxygen content and the oxidation-reduction potential are so intimately related, that observations like the one just mentioned may be misleading. The problem is yet to be investigated. The effect of oxygen and the effect of the potential must be segregated and studied separately.

The question may also be asked: what about aerobic bacteria? Is their growth controlled by the oxidation-reduction potential, or by the oxygen content of the medium? Do, for instance, anaerobic and aerobic spore formers constitute two fundamentally different classes of bacteria, or do they form a single series, the members of which differ from one another by a maximum and a minimum oxidation-reduction potential, or by a maximum and a minimum oxygen content of the medium?

The present investigation deals with the question in one of the aerobic spore-forming bacteria, *Bacillus megatherium*.

EXPERIMENTAL PROCEDURE

Our line of attack was first to familiarize ourselves with the oxidation-reduction peculiarities of certain common media under atmospheric, as well as under reduced, air pressure, and to study the conditions under which *Bacillus megatherium* grows in these media. The media we used most frequently were meat infusion broth and ordinary nutrient broth, but we have often used various synthetic media and peptone solutions.

Then we sought to influence the potential of our media by adding various oxidizing or reducing substances, so that their potential could be raised or lowered as independently from their oxygen content as possible. We sought reducing substances which, when added to a culture tube, would reduce its potential, in the air, below a certain figure found to correspond to inhibition of growth under vacuum, and oxidizing substances which would give the medium, under a known inhibiting vacuum, a potential superior to that certain figure which, in the normal medium, corresponds to inhibition of growth. These oxidizing and reducing substances must be of low toxicity so that they may be added in concentrations sufficient to produce the desired degree of shift in potential.

TECHNIQUE AND APPARATUS

Our potential measurements were made in the usual way with a potentiometer with respect to the saturated calomel electrode. We used a platinum electrode made of fine wire (gauge 30). A short piece of wire was sealed by both ends to a glass tube, thus forming a loop. Connection between the culture tube and the calomel electrode was made by means of a saturated potassium-chloride-agar bridge dipping into a tube of saturated, aqueous potassium chloride into which dips also the side tube of the calomel half-cell. The complete cell can be represented as follows:

Hg/HgCl / Saturated KCl / Saturated KCl agar / medium / Platinum

The medium under investigation was placed in a test tube of 21 to 22 mm. internal diameter, so that a 10- cc. portion formed a column of 31 to 33 mm. In this way we reduced considerably the

errors which may result from the position of the electrode in the medium, due to differences in oxygen content between liquid layers at different levels.

In order to measure the potential under reduced pressure, the above cell was mounted on a square, shellacked wooden test tube block and placed inside of a pyrex vacuum desiccator, and connection with the potentiometer was made by means of copper wires through a rubber stopper. The outfit was arranged so that two medium half-cells could be switched alternately into circuit with the calomel half-cell. In this way all our experiments were run in duplicate.

In addition to the wire leads, the rubber stopper carried also a thermometer and a small, closed-arm manometer which enabled us to read the temperature and pressure inside of the desiccator irrespective of the atmospheric pressure. The manometer had a side arm, closed with a glass stopcock, through which evacuation was carried.

The test tube block carried also one or more tubes of distilled water for the purpose of maintaining a known vapor pressure inside of the desiccator, and two tubes of the medium under investigation inoculated from a week old slant culture of *Bacillus megatherium* to test for growth. Whenever the medium investigated was not ordinary meat infusion, two more tubes of similarly inoculated normal meat infusion were also added for comparison.

I. The oxidation-reduction potential of sterile, normal meat infusion broth

Although one finds in the literature data on the oxidation-reduction potentials of culture media, we found it desirable to secure first-hand information about the oxidation-reduction properties of the media we used, both in the air and under vacuum.

In this, as in other cases, the drift in potential readings was recorded, but we considered the true potential of the medium a certain constant value obtained after a variable length of time. Our zero time refers merely to the first reading taken immediately after the outfit was assembled.

In the air the potential of the sterile meat infusion broth is

nearly equal to that of the saturated calomel electrode, usually a few millivolts more negative. Under reduced air pressure, the potential drops considerably with the pressure. Figure 1 contains the results of measurements made in the air and under relatively high vacuum.

Growth of Bacillus megatherium in normal meat infusion broth. In the air, Bacillus megatherium grows profusely in normal meat

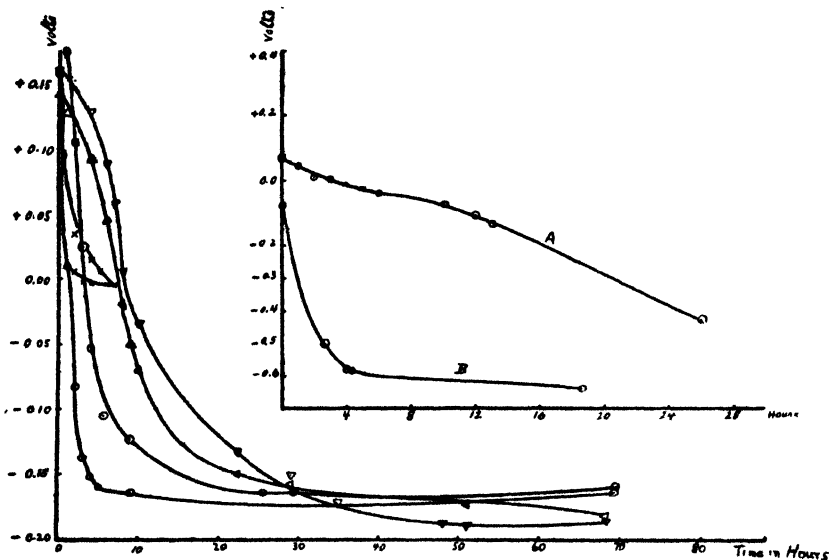


FIG. 1. Time-potential diagrams of sterile meat infusion broth under atmospheric and reduced pressure, and of meat infusion broth inoculated with *Bacillus megatherium*. Readings are with respect to the saturated calomel electrode. \times = atmospheric pressure; \circ = 20 mm. of air pressure; ∇ = 15 mm. of air pressure. Insert: A = inoculated with a loopful of cell suspension. Incubated under an air pressure of 330 mm. Hg. at 26°C. B = heavily inoculated. Incubated under atmospheric pressure at 30°C.

infusion broth of the proper reaction, pH 5.6 being the lower pH limit. If the inoculum is slight, no change in the potential of the medium is observed until growth begins. A heavy inoculum shifts the potential immediately to the negative side. As growth goes on, the potential becomes more and more negative. The diagrams inserted in figure 1 give potential readings on cultures of *B. megatherium* under atmospheric pressure and under moderate vacuum.

If the inoculated tubes be incubated in the desiccator and the air pressure within the desiccator reduced, it will be found that good growth still occurs at a considerable vacuum, and not until the air pressure inside of the desiccator has dropped below 10 mm. of mercury is growth inhibited. At this point, the potential of the medium drops to -0.160 volt or below. Even at that low pressure, microscopic examination often reveals a few young cells, although the tube may look perfectly clear. Determination of absolute limits of growth, whether it be in the anaerobic jar or in the presence of poisons, is a very difficult task, and it is our intention to study this question further.

II. *The meat infusion-sulfite medium*

In our search for a reducing substance which would reduce the potential of meat infusion below -0.160 volt under atmospheric pressure, we tried several compounds like cystein, glucose, and ferrous sulfate. None of these was satisfactory and they were dropped when, on trying sodium sulfite, we found the interesting results given below.

After having satisfied ourselves of the efficiency of sodium sulfite in reducing the potential in the air, we studied the inhibiting action and toxicity of the compound for *Bacillus megatherium*. We prepared a series of infusion sulfite media containing from 0.1 to 1.5 per cent of sodium sulfite. After sterilization of the infusion broth, the sulfite was added, and the tubes allowed to stand in the laboratory for twenty-four hours before inoculation. The idea was to allow the medium to absorb oxygen. The inoculated tubes were incubated at 30°C . and examined for growth after four days. Our results consistently showed growth in 0.25 per cent and no growth in 0.27 per cent of sodium sulfite. The higher concentrations showed growth on prolonged incubation up to 0.45 per cent, but not in 0.5 per cent or above.

These results suggest that inhibition by 0.27 to 0.45 per cent sodium sulfite is only temporary because of its reducing action, the inhibiting effect being removed on oxidation of the sulfite. Only in concentrations of 0.5 per cent and above does the sulfite become toxic, destroying the inoculum.

Potential measurements on 0.27 per cent sulfite infusion are given in figure 2, and it is seen that the potential is below -0.160 volt, the same reading obtained in normal infusion broth under inhibiting vacuum.

Was this a coincidence? or was it of fundamental significance? A clue to the mechanism was suggested to us by the observation that the potential of the 0.27 per cent sulfite infusion was the same in the air as under high vacuum. It was then that we began

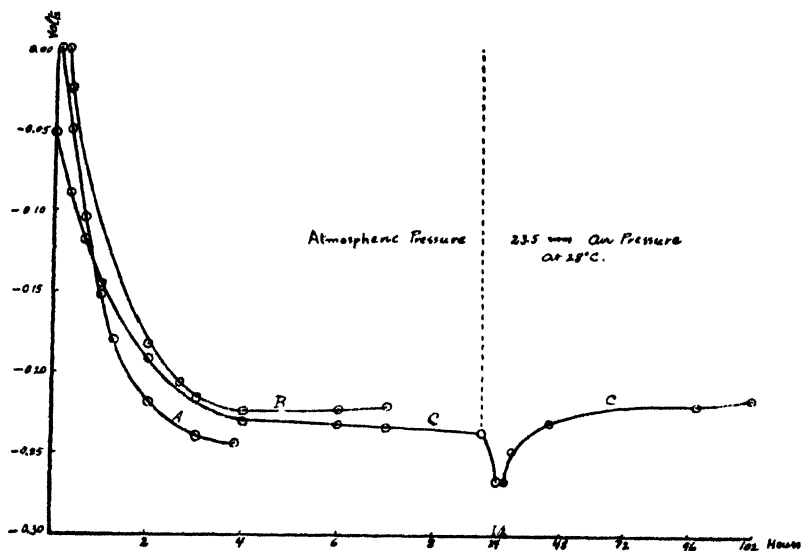


FIG. 2. Time—potential diagrams of meat infusion broth of pH 6.8, containing 0.27 per cent sodium sulfite. Readings refer to the saturated calomel electrode.

to investigate the oxygen content of our culture media as will be indicated below.

III. The ferric ammonium citrate infusion broth

In our search for an oxidizing substance which would keep the potential of the culture medium high, even under high vacuum, we spent considerable time investigating compounds like sodium nitrite, sodium nitrate, various ferric salts, 1-naphthol, 2-sulfonate indophenol, kindly furnished by Professor W. M. Clark, and litmus. Various concentrations of these compounds were tried,

but all were unsatisfactory, either because of inefficiency or because of toxicity. We also tried various synthetic media which gave us higher potentials than biological media. But the most satisfactory results were obtained from media, synthetic or biological, to which ferric ammonium citrate was added.

Ferric ammonium citrate can be added to culture media in relatively high concentrations without hindering growth. But this citrate is very sensitive to light and may give a low or a high potential, depending on whether it has or has not been exposed to

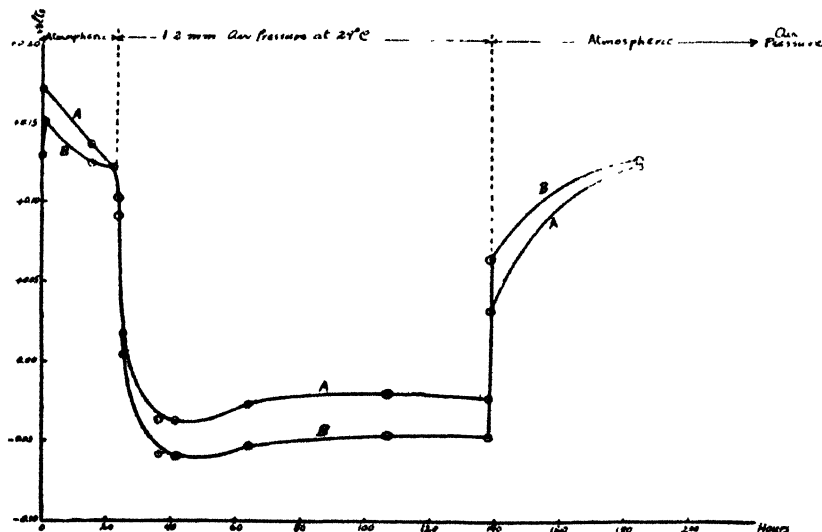


FIG. 3. Time—potential diagrams of ferric ammonium citrate nutrient broth of pH 7, protected from light during preparation and measurement. Readings refer to the saturated calomel electrode.

light. We were, therefore, very careful in preparing the medium in relative darkness and in wrapping all containers in black paper throughout the experiments. The ferric ammonium citrate media have a high potential in the air and remain considerably above -0.160 volt in the desiccator. Although we ran many experiments with synthetic media containing the citrate, most of our experiments were with ordinary meat extract broth containing 0.5 per cent of ferric ammonium citrate.

Figure 3 contains the results of a few experiments with ferric ammonium citrate media.

TABLE 1

Oxygen content of various culture media

Cubic centimeter of oxygen in 20 cc. of medium at 30°C.

MEDIUM	DUPLICATE DETERMINATIONS	AVERAGE
Meat infusion broth; pH 7	0.07 0.07	0.07
Nutrient broth; pH 7	0.08 0.11	0.095
Nutrient broth + 0.5 per cent ferric ammonium citrate; pH 7	0.12 0.12	0.12
Dox's solution + 0.1 per cent ferric ammonium citrate; pH 6	0.19 0.16	0.175
Meat infusion + 0.27 per cent sodium sulfite; pH 7	0.00 0.00	0.00
Nutrient broth made 0.05 molar ferrous potassium citrate	0.00 0.00	0.00

TABLE 2

Summary of results

MEDIUM	AIR PRESSURE	POTENTIAL	OXYGEN CONTENT IN 20 CC MEDIUM	GROWTH
Meat infusion broth	Atmospheric	volts -0.003 to -0.005	cc. 0.7	+++
Meat infusion broth	10 mm. Hg	< -0.160		-
Meat infusion broth + 0.27 per cent sodium sulfite	Atmospheric	< -0.200	0.00	-
Meat infusion broth + 0.27 per cent sodium sulfite	23.5 mm. Hg	< -0.200		-
Nutrient broth + 0.5 per cent ferric ammonium citrate	Atmospheric	About +0.100	0.12	++
Nutrient broth + 0.5 per cent ferric ammonium citrate	< 10 mm. Hg	> -0.050		-

IV. The oxygen content of various media

The results of the investigations outlined above are summarized in table 2. These results may be stated as follows:

Bacillus megatherium grows profusely in meat infusion broth of the proper reaction under atmospheric pressure. The potential of sterile, neutral meat infusion broth in the air is nearly 0.000 referred to the saturated calomel half-cell. Growth can be inhibited at about 10 mm. of mercury at 30°C., and the potential of neutral meat infusion broth at that pressure is -0.160 volt or below.

When sodium sulfite is added to meat infusion broth, the potential drops considerably, even in the air. The lowest concentration of sulfite that inhibits growth in four days is 0.27 per cent, and the potential of neutral meat infusion broth containing 0.27 per cent of sodium sulfite is about -0.250 volt in the air and does not change on evacuation.

When ferric ammonium citrate is added to ordinary nutrient broth and the medium protected from light, the potential is over $+0.100$ volt in the air and growth is profuse. On evacuation to a pressure of about 10 mm. of mercury, this potential drops to about -0.040 volt and growth is inhibited.

If the potential of the medium were the determining factor, it would not be possible to reconcile the results obtained. In normal meat infusion broth excellent growth can be obtained when the medium is given a potential of -0.040 or much below. Why is it then that no growth takes place in the citrate medium at that potential, when we know that this medium is very suitable for growth under atmospheric pressure? The fundamental difference between the two media is that a very high vacuum is required to bring the citrate medium down to -0.040 volt, while normal meat infusion reaches that potential at a relatively low vacuum. The action of oxygen is here very strongly suggested. But we have, on the other hand the 0.27 per cent sulfite medium which does not allow growth in the air, and its potential is comparable to that which is found in normal meat infusion under inhibiting vacuum.

This apparent contradiction made it indispensable to study the the oxygen content of sulfite and other media.

The apparatus used to determine oxygen was Van Slyke's blood gas apparatus, with alkaline pyrogallate to absorb the oxygen. We modified the technique to suit our purpose and used

20 cc. samples. All media were analyzed for oxygen after standing in the 30° incubator for twenty-four hours, and all oxygen determinations were made at that temperature.

The results are recorded in table 1, and they show that the sulfite medium which inhibited growth of *Bacillus megatherium* was free from oxygen or, more conservatively, its oxygen content in a 20-cc. sample was below the sensitivity of the apparatus which reads to 0.01 cc. This explains why the potential of 0.27 per cent sulfite broth is the same in the air and under vacuum.

The other figures show also an interesting comparison between the oxygen content of the other media tested. Dox's solution containing ferric ammonium citrate, which was used in some unreported experiments, contains more oxygen than nutrient citrate broth, and the latter contains more than normal nutrient broth. Normal meat infusion has the lowest oxygen content of all. Biological media are never in equilibrium with the atmosphere, they are constantly absorbing oxygen, and their oxygen consumption is proportional to their reduction potentials.

SUMMARY AND CONCLUSION

The present investigation shows that normal meat infusion broth of pH 7 has a potential nearly equal to that of the saturated calomel electrode. In a vacuum that inhibits the growth of *Bacillus megatherium* (≤ 10 mm. of air pressure), this potential drops to -0.160 volt or below.

If sodium sulfite is added to meat infusion broth, growth is inhibited when the concentration of sulfite reaches 0.27 per cent. The potential of this medium is nearly -0.250 volt in the air and does not change in the vacuum.

When ferric ammonium citrate is added to nutrient broth in a concentration of 0.5 per cent, growth takes place readily. In the air, the potential of such a medium is over $+0.100$ volt, and about -0.040 volt under a vacuum that inhibits the growth of *Bacillus megatherium*. Even at that relatively high potential of -0.040 volt, the organism does not grow.

The oxygen contents of the above media, after standing for twenty-four hours at 30°C., were in cubic centimeter of oxygen in

20-cc. samples as follows: meat infusion broth of pH 7, 0.07 cc.; meat infusion broth +0.27 per cent sodium sulfite, 0.00 cc.; meat extract broth +0.5 per cent ferric ammonium citrate, 0.12 cc.

These results show that the limiting factor in the growth of *Bacillus megatherium* in vacuum is the oxygen content and not the oxidation-reduction potential of the culture medium.

STUDIES ON DISSOCIATION OF CERTAIN PARATYPHOID BACILLI

THE RÔLE OF VARIANTS IN THE PRECIPITATION OF CALCIUM SULPHITE

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Although bacterial variation has attracted attention since the early days of bacteriological investigations, interest in this field in recent years has been greatly stimulated by the work of Arkwright (1921), Schütze (1921), de Kruif (1921) and others. Since this literature has been comprehensively reviewed by Hadley (1927, 1931a) an attempt will be made to avoid repetition. This paper deals with what are thought to be variant cells which have the ability to precipitate calcium sulphite in a suitable environment; these have arisen under conditions conducive to dissociation and, as far as the author is aware, have not been reported. In some respects they seem to be related to the familiar variants of usual colony forms of certain bacteria under enforced dissociation.

In the course of bacteriophage studies, sealed filtrates of *Salmonella Schottmülleri* frequently became opalescent or cloudy. Such observations have been reported by Hauduroy (1927), d'Herelle et al. (1930) and others on certain bacterial filtrates. Agar plates streaked from these *S. Schottmülleri* filtrates gave rise to very small, opaque bodies (fig. 1) which were superimposed upon and at times present in the medium surrounding ordinary colonies. These will be referred to as thiosomes which, due to their opacity, were black by transmitted light, white by reflected light, showed extinction with crossed nicols and were iridescent with polarized light. Apparently associated with the thiosomes were more typically daughter-colony-like structures herein re-

ferred to as atypical thiosomes (figs. 4 and 6). These were devoid of blackness, varied in their degrees of translucency, were not anisotropic and never occurred outside the colony. Upon a synthetic medium and upon ordinary fresh veal infusion medium only atypical thiosomes appeared in the colonies.

Under different conditions, more or less obscure, thiosomes exhibited variations as to size, shape, occurrence, relationship with R (rough) and S (smooth) colonies and reactions to different media. For example, in size, some resembled the finest of soot particles, while others were regarded as small, as intermediate or as large, when viewed with a colony microscope ($\times 40$), i.e., they varied in diameter from 0.005 to 0.015 mm. Those of the soot-like type were sometimes heaped upon a colony to such an extent that the margin of the latter was obscured; again, they were confined to a definite area on the colony, for instance, forming a submarginal zone. Within this zone in the clear central region large thiosomes were frequent and the majority of these were distinctly thick, structure-possessing bodies. However, the large and smaller thiosomes with no apparent patterns were most common. Frequently, all sizes were intermingled. In shape, the majority of thiosomes were spherical and not infrequently two of these were so closely united that they were termed diplococoid-thiosomes. Elliptical forms were not uncommon and when of sufficient size, their internal structures were of interest. Thiosomes at times were associated with every colony on an agar plate; sometimes, however, they appeared only with the S or Sr colonies, certainly infrequently with the moderately R type; if they were few in number they occurred only with the growth arising from the initial streak of an inoculating needle. In serial transfers, with little or no indication, they frequently disappeared temporarily,—to return suddenly in small or great numbers. The isolation or crowding of colonies on a plate, as well as their inherent vigor, often influenced the picture. Whenever colonies exhibited the possibility of bacteriophage activity, thiosomes did not appear in the region of the "bitten" areas. Upon making a Gram stain of a smear from an area containing a thiosome, small, highly refractive granules were seen along with the Gram-negative

bacilli; impression preparations were made of thiosomes and one is shown in figure 2; if the cover slip was tapped lightly, when making an impression preparation, the thiosome ruptured and the embedded granules escaped from their confinement within a capsule-like structure (fig. 3).

Conditions for the occurrence of thiosomes were essentially those which were conducive to dissociation. Media used contained several concentrations of NaCl and CaCl₂ and mammalian Ringer's; media to which no salts were added were also used. All of these will be described for each particular series or problem. Emphasis must be placed upon the two kinds of veal infusion media: fresh veal and a dehydrated Bacto-veal infusion. Upon chemical analysis the latter contained 1.5 mgm. of calcium per liter, while the former showed only a trace.¹ Qualitative microchemical analyses were made to detect cations and anions present in both the typical and atypical thiosomes. The formation of thiosomes was influenced by media whose nutritive qualities varied as is shown by a comparison of the atypical thiosomes on a synthetic medium or on fresh veal media with the typical ones when Bacto-veal infusion or fresh veal infusion to which calcium was added was used as a base. The studies of Jordan (1926) and others, relative to interconvertibility of R and S types suggested that rapid transfers be employed and these exhibited much of interest not only in direct relation to thiosomes but also permitted of a correlation between these structures and certain growth characteristics in broth such as surface growth, turbidity, sediment and motility.

The microchemical technique employed for the detection of cations and anions present in the thiosomes will be described later. Figures 6 and 7 illustrate crystals typical of Ca⁺⁺ and SO₃⁻⁻.

Problems relative to the sources and characteristics of thiosomes and their correlation with cultural features will be treated in the following order:

¹ The author is indebted to Mr. R. A. Green, Assistant Agricultural Chemist, University of Arizona, for the determination of the calcium content of these two infusions.

Series I. Cultural studies of *S. Schottmülleri* filtrates in salt agar and bouillon.

Series II. Thiosomes from *S. Schottmülleri* in serial salt broths.

Series III. Thiosomes from certain other members of the colony-typhoid group.

Series IV. Thiosomes from *E. coli* isolated from pathological material.

Series V. The influence of a synthetic medium upon thiosomes.²

MICROCHEMICAL ANALYSES

In an effort to identify cations and anions that might be present in thiosomes it was necessary to resort to "chemical microscopy" as described by Chamot and Mason (1931). Microscopic qualitative chemical analysis demands special technique and within certain limitations permits of results that otherwise could not be obtained. Many of the "micro" tests were not applicable to the present problem due to factors that will be mentioned in connection with individual analyses. The minimum of material available for examination must be emphasized together with the impossibility, with rare exceptions, of its separation from substances of great complexity in the colony and the medium. When tests demand careful control of such factors as temperature, acidity or alkalinity, and concentrations, they become practically impossible under the present conditions. The following factors may give some appreciation of the intricacy of the problem: (1) the complexity of the mixtures of substances in different media; (2) the factors introduced by the metabolic activities of the bacteria themselves; (3) the fewer specific tests for anions than for cations; (4) the possibility that the cation in the unknown might react with an anion in the reagent; (5) the nature of the cation might influence the behavior of an anion toward reagents.

Had it been possible to place thiosomes upon a microscope slide and use the ordinary "micro" tests of decantation, filtration, sublimation, distillations, etc., many difficulties would have been eliminated. Since adequate separation of thiosomes was impossible, the tests were carried out using thiosomes associated with colonies of varying ages on ordinary moist agar plates and also after the agar had been dried at room temperature nearly to the point of cracking. The latter plates gave more clear cut results. The plates were oriented on the stage of a

² I wish to acknowledge my indebtedness to Dr. E. O. Jordan for his assistance and stimulating interest during the progress of this work.

colony microscope and the various reagents added by means of Pasteur pipettes. Magnifications of 40, 100 and 440 diameters were ordinarily used. Identification of cations was carried parallel to the identification of anions. In general, the Bunsen-Treadwell classification of the acids was followed. "This classification is based upon the behavior of the commoner anions toward silver nitrate and toward barium chloride in neutral and in nitric acid solutions" (Chamot and Mason, 1931). Although this particular problem presented certain difficulties, peculiar perhaps to the usual application of microchemical analysis and was consequently subject at times to some limitations, nevertheless the value of such technique should be emphasized. Used singly, tests are often inconclusive but together they permit of the identification of ions with a remarkable certainty and dependability.¹

Detection of anions. Tests which indicated the absence of borates, nitrates, nitrites, mono-, di- or tri-alkali phosphates together with hypophosphites and phosphites are not included in this paper because of the limitation of space. There were probably no carbonates of Na and of the K group or most of the other elements present, but due to technical difficulties and the extremely minute amounts of gas evolved when acids were applied, even though no crystals or aggregates were produced, the presence of carbonates could not be completely ruled out. One of the tests suggested orthophosphates but this could not be confirmed with other reagents. According to Chamot and Mason (1931), "salts of SO_4^{--} are seldom contaminated with salts of the other ions of the group, but the salts of the remaining ions ordinarily contain more or less impurities, such as sulphates or salts of the other more closely related ions, the nature of which depends upon the method used for the formation of the ion in question. These foreign ions, which are almost invariably present, introduce a complication in the analysis of very small amounts of material and may trouble the analyst." SO_4^{--} was occasionally indicated in some thiosomes and SO_3^{--} was regularly revealed by the group reactions. BaSO_3 crystals were readily soluble in HCl and typically anisotropic. $\text{Ca}(\text{C}_2\text{H}_3\text{O}_2)_2$ produced in plates, which did or did not contain thiosomes, tiny disks and spherulites which tended to develop into aggregates. These formed slowly and attained the approximate size of thiosomes. At first, these spheres developed in the medium around the colony margin but after thirty to sixty minutes

¹ The author gratefully acknowledges the assistance of Dr. M. N. Short, Professor of Optical Mineralogy, University of Arizona, who verified the identity of the crystals.

they occurred distributed through out the colony area and were almost as large as but less numerous than the thiosomes. These artificial thiosomes or CaSO_3 crystals appeared identical with all previously described thiosomes and where Ca^{++} and SO_3^{--} concentrations were high artificial soot-like thiosomes simulated those produced by *S. Schottmülleri* on CaCl_2 agar plates. Thus, the formation of artificial thiosomes upon addition of calcium clearly indicated the presence of an excess of SO_3^{--} due to bacterial activity. Therefore, calcium present only to the extent of 1.5 mgm. per liter, in Bacto-medium, was insufficient to unite with all the SO_3 ions. The above results were obtained with the Bacto-veal infusion medium without the addition of any salt and also with the NaCl plates. When the above was repeated using the uninoculated control plates no artificial thiosomes occurred. When $\text{Ca}(\text{C}_2\text{H}_3\text{O}_2)_2$ was added to colonies on $\text{m}/10$ NaCl agar which had fresh veal infusion as a base and hence possessed no thiosomes, artificial thiosomes developed which were identical to those on the Bacto-agar plates (fig. 6). These occurred primarily around the margin of the colony but there were also a few scattered spheres in the central areas of colonies. As a rule, one to two hours was required for appearance. It was of interest to note that in some plates sulphite crystals formed a compact zone around the margin of the colony provided the latter was well isolated; if the colony margin was near another colony, then the artificial thiosomes were scattered and irregular in the peripheral region. This response to crowding corresponded to the way in which the "Schleimwallbildung" phenomenon was noted. Thus, SO_3 ions were present as a metabolic by-product but with insufficient calcium present in fresh veal, which upon analysis showed only a minute trace of calcium, no precipitate could form. When the above was repeated using $\text{m}/10$ NaCl agar which had fresh veal infusion as a base and to which phosphate had been added, using a pH of both 7.2 and 7.8, no artificial thiosomes formed. Does this mean that the presence of phosphate inhibits sulphite crystal formation? Since dibasic potassium phosphate is often added as a buffer and since the commonly used fresh veal has a comparatively high phosphate content, these two factors may account for the failure to date to find these sulphite crystals reported in the literature. By the $\text{Ca}(\text{C}_2\text{H}_3\text{O}_2)_2$ test, sulphites may be distinguished from thiosulphates since the calcium salt of $\text{S}_2\text{O}_3^{--}$ is too soluble to separate. Thus, this serves as another means of confirming the presence of CaSO_3 . Thiosulphates and persulphates were not detected.

Detection of cations. The chemical tests which established the identity of the predominant anion, namely, sulphite, also suggested calcium as the cation. Tests for many of the other elements likely to be present either proved definitely negative or else the difficulties already pointed out entered to such an extent as to exclude, at least until technique can be further developed, the presence in sufficient quantity of any other element which might play a definite rôle in the formation of thiosomes. Although there is a close relationship between the alkaline earths generally, there are differences in the appearances and solubilities of their salts which permit of differentiation.

Promptly upon addition of sulphuric acid effervescence within the thiosomes occurred. Different concentrations of H_2SO_4 were used and within rather wide limits the same crystals were obtained. After the bubbles had risen to the surface of the test drop, the thiosome appeared to have lost something of its opacity and often highly resembled a translucent or atypical thiosome (fig. 5). The latter was especially true when a concentration of $\text{m}/5 \text{H}_2\text{SO}_4$ was used. After about ten to fifteen minutes the thiosome appeared somewhat fuzzy and crystals typical of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ rapidly formed (fig. 7). Long monoclinic prisms formed with ends which became irregularly and obliquely truncated. Sometimes these prisms gave rise to plates or scales which is a characteristic of Ca^{++} . Photographic records of these changes were carefully made and controls were run with various uninoculated media. Since this test gave such typical crystals, salts of any trivalent metals were excluded, for had these been present they would have interfered with this sulphuric acid test. Although strontium, barium and lead yield crystalline precipitates these could be ruled out with certainty, because of lack of rapid disintegration into a granular deposit, absence of an amorphous precipitate and general characteristics as to appearance and solubilities respectively. The presence of calcium was confirmed by means of the oxalic acid test.

Attempts were made to identify calcium by means of the following reagents: (1) sodium tartrate strongly acidified with tartaric acid, (2) potassium ferrocyanide, and (3) arsenic acid. None of these were satisfactory. This is not surprising since these tests require a moderately high concentration of Ca, which is higher, evidently, than was present in thiosomes; also, since the acidity or alkalinity is of great importance this constituted a factor most difficult of control. Only with the CaCl_2 uninoculated control plates were crystals typical of calcium obtained.

SERIES I. THIOSOMES FROM *S. SCHOTTMÜLLERI*, R TYPE, FILTRATES

In certain bacteriophage studies, carried on with a single-cell culture of *S. Schottmülleri*, 210R₁,⁴ Bacto-veal infusion bouillon and agar,⁵ pH 7.4 and having the following salt concentrations were used: M/1 NaCl, M/2 NaCl, M/10 NaCl, mammalian Ringer's, M/5 CaCl₂, M/10 CaCl₂ and H₂O. The "H₂O" broth or agar simply denotes the total absence of added salts. The 210R₁ culture had been well adapted to these salt concentrations since during the previous eight months it had been used in studies relative to certain dissociative phenomena. In an attempt to ascertain why a relatively high percentage of Berkefeld filtrates in sealed glass ampoules became more or less cloudy or opalescent, the ampoules were well shaken, opened and 5 drops of each transferred to the respective salt broths. From the twenty- to twenty-four-hour-old salt broth cultures, incubated at 37°C., daily motility tests were made, smears for Gram stains prepared and salt agar plates streaked.

Two examples of the characteristics of daily transfers in two salt broths, M/10 NaCl and M/10 CaCl₂, may be briefly summarized as follows: In both media, changes in the surface growth occurred about the 6th transfer and again upon the fourteenth or fifteenth transfers; turbidity reached a maximum on the fourth day and maintained this maximum throughout the remainder of the experiment; the sediment was usually granular and slightly viscid until the seventh to twelfth transfer, inclusive, when it

⁴ Dr. E. O. Jordan's collection.

⁵ Bacto-veal infusion medium, dehydrated, contains veal, infusion from 500 parts; Bacto-peptone, 10 parts; Bacto-agar, 1 part; and the infusion is made in the presence of 1 per cent gelatin. Hitchens (1921) suggested certain advantages of approximately the above medium which was modified by The Digestive Ferments Company, Detroit, Mich. Since in the present investigation it was deemed undesirable to have as much agar present, the dehydrated medium was put into solution by autoclaving, 15 pounds for fifteen minutes, the flasks were placed in the refrigerator over night in order to solidify the agar and the supernatant was carefully filtered through a combined paper and cotton filter. The infusion was made double strength, an equal volume of double strength salt solution was added, titrated, tubed, sterilized (autoclaved, 15 pounds for 20 minutes), and the reaction checked. Agar media contained 2 per cent agar. All media were stored in the refrigerator and care taken to avoid evaporation.

lost its viscosity. The latter returned to a slight degree on the thirteenth, fourteenth and fifteenth transfers; about this time the sediment was also rather flocculent. At about the sixth and again at about the twelfth or thirteenth transfers there was a decrease in the number of motile cells in the $m/10$ NaCl medium which may also be correlated with findings recorded under series II. The $m/10$ $CaCl_2$ showed in both series considerable fluctuations in motility.

In $m/10$ NaCl, upon first transfer, large, reddish-purple structureless, refractile, coccoid bodies predominated. These became elongated, and large, rather granular, Gram-negative rods subsequently appeared. Pleomorphic forms decreased in numbers in the course of the series of broth transfers and eventually gave rise to text-book-like pictures of *S. Schottmülleri*. As had previously been noted, thiosomes sometimes disappeared for one or more consecutive transfers, suddenly to appear again, and it is of interest to note here that thiosomes were seen in the Gram-stained smear from the broth transfer which preceded the finding of thiosomes on agar plates. It should also be mentioned here that familiarity with these pleomorphic cells permitted the recognition of similar forms, which will be subsequently reported, in connection with the finding of thiosomes associated with an atypical colon bacillus isolated from a case of cervicitis. In $m/10$ $CaCl_2$, upon first transfer, large Gram-positive coccoid bodies, which varied in size and shape, predominated. These bodies subsequently were less deeply stained and with them were seen some large Gram-negative rods together with thiosomes which showed the capsule with its internal refractive granules (fig. 2). Cells in these broth transfers were subject to great variations in staining reactions and in morphology, but finally all became definitely Gram-negative; some continued to be somewhat swollen and irregular but most of them were typical of *S. Schottmülleri*.

Thiosomes appeared on the plates streaked from the first 18 transfers in $m/10$ NaCl broth upon the following days: first, second (not until forty-eight hours incubation), seventh, eighth, ninth (fig. 8), twelfth, seventeenth and eighteenth. This illustrates the irregularity of their appearance especially in the pres-

ence of NaCl, which, together with the changes in growth characteristics in broth, is simply further evidence that bacterial protoplasm is not stable, and that its responses to varying conditions must be studied to a greater extent in their entirety before the probable phases in growth become better understood. Serial transfers constitute one means of accelerating changes that, in the recent past, have gone unrecognized largely due to their suppression because of methods not favorable to dissociation. The thiosomes in $M/10$ NaCl were never very numerous but definitely well developed. From $M/10$ $CaCl_2$ broth, thiosomes were present in every transfer. They were always well developed and abundant in numbers except in the fourth transfer when they failed to appear until forty-eight hours of incubation and were then relatively few in number. The thiosome-colony relationship was very marked, with huge thiosomes superimposed upon the colony in comparison with the small thiosomes in the medium. No elongated thiosomes were seen but the spherical, diplococcoid type was most abundant in the medium surrounding the colonies. From none of the filtrates did the previously mentioned soot-like thiosomes arise.

Attention is called to the thiosomes with the scalloped margins (fig. 8), which have lost their blackness and are less opaque. Thus far, thiosomes superimposed upon colonies only have presented such a picture, but if such a type could be found in the medium surrounding a colony, an impression preparation might reveal more of the actual structure than is seen in preparations more or less confused by ordinary colony cells. When the calcium sulphite is dissolved by suitable reagents, a colony-like structure remains, devoid of any blackness, and with the scalloped margins intact. Whether the bacterial cells that compose this type of margin develop from the colony itself in response to the presence of this crystalline mass, or whether they are the type of cell presumably capable of precipitating $CaSO_3$ when a sufficient number of such ions are available, remains unanswered. In other words, perhaps the thiosomes are in reality daughter-like colonies.

SERIES II. THIOSOMES FROM *S. SCHOTTMÜLLERI*, R TYPE, IN
SERIAL SALT BROTHS

In the course of certain bacteriophage studies, Bacto-veal infusion agar plates (m/10 NaCl, pH 7.4 to 7.6) were streaked from the daily transfer of 210R₁ in salt broths for the purpose of making observations upon the relative percentages of S, Sr, sR or R colonies which might develop from day to day. These broths contained the same salt concentrations used in series I. The first four daily transfers gave rise to colonies from each broth that were S or Sr in type and of no special interest. No plates were streaked from the fifth, sixth or seventh transfers but upon examination of twenty-hour-old plates from the eighth transfer, extremely numerous thiosomes were seen on all seven plates. Thiosomes appeared upon all plates from the ninth and tenth transfers and two of the latter are illustrated (figs. 9 and 10). Control plates on the media were made and carefully observed concurrently with all transfers but no thiosomes ever appeared upon these sterile plates.

In addition to general characteristics of thiosomes previously given, it was noted that size decreased with either an increase in NaCl concentration or with crowding of the colonies. In shape, the spherical or nearly spherical form predominated and often appeared as a diplococcoid structure (fig. 9). The elliptical thiosomes were not uncommon and were probably most clearly observed from Ringer's broth. It was unusual to find as well developed thiosomes in the agar as are pictured in figure 9. If thiosomes were extremely few in number, they were found where streaking of the plate was begun, i.e., following the line of the first needle tract and here, only, on the entire plate. Occurrence, especially from CaCl₂ bouillon, of very large thiosomes around the periphery of the colony resulted in a perforation of the submarginal area, or an indentation of the edge (fig. 10).

After the appearance of thiosomes upon the eighth transfer, they persisted through the twelfth transfer, but upon the thirteenth transfer they disappeared from certain plates. Observations made upon the thirteenth to eighteenth transfers inclusive are given in (table 1).

TABLE 1

Presence or absence of thiosomes from S. Schottmüller, thirteenth to eighteenth daily salt broth transfers

TRANSFER	M/1 NaCl	M/2 NaCl	M/10 NaCl	RINGER'S	M/5 CaCl ₂	M/10 CaCl ₂	H ₂ O
13th	—	— ¹	+ ²	— ³	+ ⁴	+ ⁵	+ ⁶
14th	+	+	+	++	+++ ⁷	+++ ⁸	+++ ⁹
15th	— ¹⁰	— ¹¹	++ ¹²	+ ¹³	+++ ¹⁴	+++ ¹⁵	+ ¹⁶
16th	+ ¹⁷	+ ¹⁸	+ ¹⁹	+ ¹⁹	+ ²⁰	— ²¹	+ ²²
17th	— ²³	— ²³	++ ²⁴	++ ²⁵	+++ ²⁵	+++ ²⁶	++ ²⁷
18th	+ ²⁸	+ ²⁸	++ ²⁹	++ ²⁹	+++ ³⁰	+++ ³¹	++ ³¹

¹ Myriads of shadow-like colonies; small, round, highly transparent, entire margins but rough surfaces.

² Thiosomes only upon initial streaks on plate.

³ S and few Sr colonies.

⁴ S and Sr colonies predominated; few Rs and R.

⁵ Fewer thiosomes than from m/5 CaCl₂; also fewer R type.

⁶ Many shadow-like colonies but not as small or as transparent as from m/2 NaCl; mostly S and Sr. Thiosomes only on initial streaks.

⁷ Some shadow-like colonies like m/2 NaCl, thirteenth transfer.

⁸ Thiosomes covered plate; largest perforating thiosomes on initial streaks.

⁹ Few perforating thiosomes.

¹⁰ S and Sr colonies.

¹¹ Sr colonies.

¹² S and Sr colonies. Thiosomes associated with about half of colonies.

¹³ S and few Sr colonies. Thiosomes only on initial streaks.

¹⁴ S, Sr, and R, with tendency toward latter.

¹⁵ Few S; sR and R predominate. Fewer thiosomes than from m/5 CaCl₂. Perforating thiosomes along initial streaks.

¹⁶ S, few sR. Large thiosomes along initial streaks.

¹⁷ S, Sr, few R. Thiosomes along initial streak only.

¹⁸ S, Sr, few Rs. Thiosomes along initial streaks only.

¹⁹ S, few Sr, and many small colonies.

²⁰ S and Sr colonies.

²¹ S, Sr and few R colonies.

²² Sr and sR predominated; some thiosomes in medium adjacent to Rs colonies.

²³ S and Sr colonies.

²⁴ Very thin thiosomes often in clumps. S and Sr colonies.

²⁵ S, Sr few R. Tiny thiosomes in sheet-like growth.

²⁶ R and Sr predominated; few S thiosomes as note 25.

²⁷ R and sR predominated with very few S.

²⁸ S, few Sr. Atypical thiosomes.

²⁹ Like "28" only more numerous atypical thiosomes.

³⁰ Few opaque thiosomes but numerous atypical ones.

³¹ No opaque thiosomes but numerous atypical ones.

Throughout series II, broth characteristics were noted as to surface growth, turbidity, sediment, Gram stains and motility. Since the data accumulated from observations of 21 daily transfers in seven salt broths were so extensive they can only be taken up briefly in the discussion of this paper. Certain of these observations were of interest since they may be correlated more or less closely with each other and with other phases of dissociative phenomena.

In this series as well as others, zones or strata appeared occasionally in old broth cultures. The septa were of a membranous nature, not at all easily disturbed upon agitation, and were not present in M/1 NaCl or in either of the CaCl₂ concentrations. Even though flasks containing the dissolved Bacto-veal infusion medium were placed in the refrigerator for twelve hours or longer, and the extremely low content of solidified agar subsequently removed by filtration, nevertheless there doubtless remained a small amount of agar, so small that it perhaps formed a gel (Hitchens, 1921) which upon standing contracted and settled to the bottom of the tubes. Thus, the layering effect might have been due to growth of bacteria on the surface of the gel alone, or in some instances perhaps a slight pellicle formed and sank to this layer. However, a layering effect was noted in occasional transfers of certain series which, in connection with other work, were made in ordinary broth prepared from fresh veal. Although little has been done thus far, there is an indication that from each zone different types of colonies developed on agar plates when streaked from the upper and lower layers respectively.

SERIES III. THIOSOMES FROM CERTAIN OTHER MEMBERS OF THE COLON TYPHOID GROUP

An attempt was made to find thiosomes in association with certain other members of the colon-typhoid group. The following stock cultures were chosen: *E. coli* "D," 9 *E. typhi*, 131 *S. paratyphi*, 210 *S. Schottmülleri*, and 329 *S. paratyphi*, type C. All were of S type. Fifteen daily transfers were made in two salt media: M/10 NaCl and M/5 CaCl₂ Bacto-veal infusion bouillon and M/10 NaCl and M/5 CaCl₂ Bacto-veal infusion agar (2

TABLE 2

Presence or absence of thiosomes from five members of the colon-typhoid group during fifteen daily transfers

TRANSFER	m/10 NaCl					m/5 CaCl ₂				
	D	9	131	210	320	D	9	131	210	320
<i>1st</i>						++			++	++
24 hours	+++ ¹	-	-	-	-	++	-	-	++	++ ²
36 hours	+++	-	-	-	+ ³	++	++ ⁴	+ ⁴	++	++
						++ ⁴			++ ⁴	++ ⁴
<i>2nd</i>						++			++	++
24 hours	+++	-	-	-	+	++	+ ⁵	- ⁶	++	++
48 hours	+++ ⁷	-	-	-	+ ⁸	++	+	+	++	++
						++			++	++ ⁹
<i>3rd</i>						++			++	++
20 hours	+++	-	-	-	-	++	- ¹⁰	- ¹¹	++	++
48 hours	+++	-	-	-	-	++	+++ ¹²	-	++	++
						++			++	++
<i>4th</i>						++			++	++
20 hours	+++	-	-	-	-	++	+++	+ ¹³	++	++
48 hours	+++	-	-	-	-	++	++	++	++	++
						++	++		++	++
<i>5th</i>						++	++		++	++
24 hours	+++	-	-	-	-	++	++	-	++	++
48 hours	+++	-	-	-	+ ¹⁴	++	++	-	++	++
						++	++		++	++
<i>6th</i>						++	++		++	++
20 hours	++ ¹⁵	-	-	-	-	++ ¹⁶	++	- ¹⁷	++	++
48 hours	++ ¹⁸	-	-	-	-	++	++		++	++
						++ ¹⁹	++ ²⁰	+ ²¹	++	++ ²²
<i>7th</i>							++			++
24 hours	- ²³	-	-	-	-	+++	++	-	+++	++
48 hours	-	-	-	-	-	++	++	+ ²⁶	+++	++
						+ ²⁴	++			++
<i>8th</i>							++		++	++
20 hours	+ ²⁶	-	-	-	-	+++	++	-	++	++
48 hours	+ ²⁷	-	-	-	-	++	++	+	++	++
						+ ²⁷	++		++	++

TABLE 2—Continued

TRANSFER	m/10 NaCl					m/5 CaCl ₂				
	D	9	131	210	329	D	9	131	210	329
9th										
24 hours	+ ²⁹	—	—	—	—	+++	+++	— ³⁰	+++	+++
48 hours	+	—	—	—	—	++	++	—	++	++
						++	++		++	++
10th						++			++	++
24 hours	—	—	—	—	—	++	+++	—	++	++
48 hours	—	—	—	—	—	++	+++	—	++	++
						++			++	++
11th						++			++	++
24 hours	—	—	—	—	—	++ ³¹	+++	—	++	++
48 hours	+ ²⁹	—	—	—	+	+++	++	—	++	++
							++ ³²		++	++
12th						++			++	++
24 hours	++ ³³	—	—	—	—	++	+++	—	++	++
48 hours	+++	—	—	—	—	++	++	—	++	++
						++	++		++	++
13th						++	++		++	++
24 hours	+	— ³⁴	— ³⁴	— ³⁵	— ³⁵	++	++	—	++	++
48 hours	+ ³⁶	—	—	+ ³⁷	+	++	++	—	++	++
						++	++		++	++
14th						++			++	++
24 hours	+ ³⁸	— ³⁹	—	—	—	++	+++	—	++	++
48 hours	+	+	—	+	—	++	+++	—	++	++
						++			++	++
15th						++			++	++
20 hours	+ ³⁹	— ³⁹	— ³⁹	— ⁴⁰	— ⁴⁰	++	+++	—	++	++
48 hours	+	+	—	++	++	++	++	—	++	++
						++	++ ⁴¹		++	++

¹ S and few R colonies; latter devoid of thiosomes.

² Finest thiosomes yet observed, i.e., the colonies appeared to be buried in soot.

³ Two colonies only possessed thiosomes.

⁴ Innumerable, fine opaque thiosomes, soot-like.

⁵ Numerous soot-like thiosomes on one small area only.

⁶ Colonies very small and relatively few in number.

⁷ All colonies smooth from all five cultures on NaCl agar.

⁸ Thiosomes on only six colonies.

TABLE 2—Continued

- ⁹ Margins of crowded colonies obscured by soot-like thiosomes.
- ¹⁰ Colonies much smaller but relatively numerous.
- ¹¹ Colonies extremely small and few in number in subsequent transfers.
- ¹² Some colonies with large thiosomes, some buried in soot, some which combine previous two types and some devoid of any thiosomes.
- ¹³ Two areas only with dense fine thiosomes.
- ¹⁴ Thiosomes large, elongated and as diplococcoid bodies.
- ¹⁵ Sudden increase in R colonies (one-third of total, fig. 11). Truly R type devoid of thiosomes but a few Rs colonies possessed them.
- ¹⁶ Increase in size and structural changes of thiosomes associated with *E. coli* when seventy-two hours old (fig. 12), and the identical area after twelve days at 37°C. (fig. 13). Generally, there was little or no increase in size on NaCl or H₂O plates after thirty-six to forty-eight hours.
- ¹⁷ Marked differences in size of colonies. In forty-eight hours, thiosomes on large Rs or Sr colonies only (fig. 15, $\times 40$).
- ¹⁸⁻²³ Figures 11 to 16, inclusive, seventy-two hours, $\times 40$.
- ²⁰ Figure 14, *E. typhi*, seventy-two hours. Increase in number of soot-like thiosomes but some colonies strikingly free from them.
- ²¹ Figure 15. *S. paratyphi*, seventy-two hours, $\times 40$. Thiosomes grouped in only few areas on plate. Note translucent and semi-transparent thiosomes among typical opaque ones. Large and small colonies usual at this period and thiosomes only in conjunction with former which were also somewhat granular.
- ²² Seventy-five per cent colonies rough.
- ²⁴ Figures 17 and 18, $\times 40$. Two typical areas on this plate. Upon this transfer, decrease in relative numbers of thiosomes on CaCl₂ medium but disappearance in NaCl medium. Attention is directed to effect of crowding on thiosomes (fig. 18).
- ²⁵ R and S colonies present; R or Rs colonies only possessed thiosomes. This the only exception found where roughness was not associated with a definite decrease in thiosomes.
- ²⁶ Figure 19.
- ²⁷ Six colonies only with thiosomes.
- ²⁸ After ten days, characteristic daughter colonies; these not uncommon in other old cultures.
- ²⁹ Majority of colonies very rough.
- ³⁰ All colonies uniformly small and smooth.
- ³¹ Figure 20, seven days old. Note atypical thiosomes.
- ³² Similar to figure 20.
- ³³ Ninety per cent R colonies; thiosomes on all types but predominate on Sr and S.
- ³⁴ Few "bitten" areas in colonies. All S or Sr type.
- ³⁵ Many "bitten" areas in colonies. All S or Sr type.
- ³⁶ As twenty-four-hour colonies Sr predominated but when forty-eight hours old, S colonies developed as outgrowths along line of inoculation in certain areas and these possessed thiosomes. In certain Rs colonies, atypical thiosomes (figs. 4 and 5; fig. 20, in submarginal zone).

TABLE 2—*Concluded*

¹¹ Figures 21 and 22. Thiosomes nearer the margin appeared larger and more markedly elliptical. Note internal structure. Purely S colonies were devoid of thiosomes and showed evidence of bacteriophage activity (fig. 22). With exception of *E. coli* all cultures, 9, 131, 210 and 329 showed probable evidence of bacteriophage activity upon this thirteenth transfer.

¹² All colonies R or Rs except two S, and latter have "bitten" areas; these "bitten" areas suggestive of bacteriophage activity were devoid of thiosomes. Many R-S colony combinations resembled the "Bombenformen" of Braun and Weil (1928).

¹³ All colonies smooth and few have "bitten" edges.

¹⁴ All S or Sr colonies; few "bitten."

¹⁵ Figure 23. S type colony margins only appear when farthest away from other colonies which region contained a few large thiosomes. This phenomenon paralleled characteristic appearance or non-appearance of "Schleimwallbildung," i.e., latter not observed in closely adjacent colonies. Note also that large type of thiosome often inhibited in its vicinity the soot-like thiosomes.

per cent), the addition of calcium serving as a control. Media were prepared as for series II but with a pH of 7.8. No record of daily broth characteristics was kept. From each twenty- to twenty-four-hour-old daily broth culture, salt agar plates were streaked and the presence or absence of thiosomes tabulated (table 2) together with notes and photographs (figs. 11 to 23) of certain findings. Observations were taken regularly at both twenty-four and forty-eight hour intervals during incubation at 37°C. Occasionally records were made of certain changes in colonies that occurred at subsequent intervals. The first transfer plates were accidentally left at room temperature, about 24°C., over night after the first twenty-four hours at 37°C. Direct prints (Buchholz and Lewis (1930)) were made of the first transfer plates and areas typical of each were ringed and photographed at a magnification of 75. Photographic records were resorted to when marked changes occurred. A magnification of 40 was used for all except the first transfer.

The presence of thiosomes in conjunction with five members of the colon-typhoid group, throughout, or at certain intervals during, 15 daily transfers and their relative number from day to day on M/10 NaCl and M/5 CaCl₂ agar plates has been of much interest. On the control CaCl₂ plates, thiosomes could be noted

upon macroscopic inspection of the thirty-six-hour-old first transfer Petri plates by the cloudy areas adjacent to and including the colonies; microscopically, thiosomes were so numerous that colony margins were more or less obscured. In the footnotes of table 2 details are given with emphasis on the type of thiosome found.

SERIES IV. THIOSOMES FROM PATHOLOGICAL MATERIAL

The finding of thiosomes in association with *E. coli* isolated from a case of cervicitis was of considerable interest and points toward further investigations that should be carried out with pathological material. This patient had been undergoing treatment for a month and was not showing as rapid improvement as was expected; it was at this time that the following work was done. During the fifth week three specimens were sent to the laboratory and the results from each, herein reported, were in agreement.

This case had been reported by a technician in a local commercial laboratory as a streptococcus infection; the clinical picture contraindicated this diagnosis in the judgment of the physician. It was found that the technician made his report solely on a Gram-stained smear from a six- to eight-hour-old brain broth culture (Rosenow's formula, 1928). To check his report, the specimens herein reported were likewise inoculated into brain broth together with certain other media: nutrient broth, glucose broth containing a piece of fresh, sterile, rabbit kidney, nutrient agar, Endo's medium and veal infusion rabbit blood agar plates (both streaked and poured). Several direct smears were also obtained from the patient and stained by Gram's method. After an eight-hour incubation period in brain broth, Gram-stained preparations revealed the presence of pleomorphic cocci; the predominating type was a huge Gram-negative diplococcus which occurred frequently in chains made up of distinct diplococci. The direct smear showed this same form together with a few typical staphylococci, a few large Gram-positive rods, and some Gram-negative coli-like bacilli. From both preparations, direct smear and brain broth, the large diplococcus was less easily decolorized by alcohol than most Gram-negative organisms; this

was checked time and again with known positive and negative cultures until no hesitancy existed in reporting it as Gram-negative. With the exception of very few typical staphylococci, nothing but coli-like rods were found in other media. Transfers to sugars gave *E. coli* characteristics. After eighteen hours at 37°C. in brain broth, very few coccus-like cells were seen and these were never in chains. On Endo plates, the characteristic metallic luster of *E. coli* developed only upon forty-eight hours incubation. Transfers from brain-broth cultures to nutrient, blood, and Endo agar plates revealed nothing but colon-like colonies with an occasional typical staphylococcus and also a few colonies made up of large Gram-positive rods. When sugars were inoculated with three slant cultures of coli-like bacilli, obtained from the atypical Endo plate colonies, one of these slants was placed upon the stage of a microscope and observed under low power. Typical thiosomes were numerous and were also present in the other two Bacto-nutrient agar slants. While this is a report upon three specimens from only one case, this came at the time when thiosomes were the objects of much interest, and offers another distinct source of calcium sulphite crystals precipitated by a member of the colon-typhoid group.

In connection with pathological material, the fact is recalled that when bacteria are isolated from the centrum of certain calculi the colon and typhoid bacilli are most frequently found (Wells, 1925). The possibility that certain variant bacterial cells may have the ability to precipitate calcium and hence be of significance in the formation of certain concretions in the animal body will be discussed briefly in this paper.

SERIES V. INFLUENCE OF A SYNTHETIC MEDIUM ON THIOSOMES

An attempt was made to determine whether or not thiosomes would develop in conjunction with R and S strains of *S. Schottmülleri* when a synthetic medium was used with the same concentrations of NaCl and CaCl₂ employed with the filtrates in series I. Single-cell cultures used were those above referred to as 210R₁ and 210S₁; the former had been cultivated for some time in Bacto-veal infusion salt broths, but the latter was merely a well-

cared for stock culture. The medium used was the "number eight"⁶ reported by Stuart (1924). It will be noted that the only possible source of sulphur ions was in the magnesium sulphate, and calcium was available only in the one type of medium. Seventeen daily transfers from broth to broth and broth to agar plates were made. Observations were made as reported for the previous series except that plates were incubated for seventy-two hours at 37°C.

The R and the S strain grew far less luxuriantly than in veal infusion broths and the colonies on plates were always small. At irregular intervals growth was so scanty that certain of the higher salt concentrations had to be reseeded from a lower concentration in which a more vigorous growth was present. Considerable data accumulated from observations on 210R₁ during seventeen daily transfers in six synthetic salt broths together with six synthetic salt agar plates, and will be summarized as follows: With the R strain, 210R₁, in several synthetic salt broths, slight changes occurred in surface growths upon the ninth to tenth transfers in M/2 NaCl (hazy type of pellicle), M/10 NaCl (for the often found hazy type was substituted a moderate, white pellicle with a granular ring) and M/10 CaCl₂ (instead of irregular variations between an entire absence of pellicle formation or the thin transparent type a slight ring pellicle developed); the ninth transfers also gave rise to a somewhat granular sediment in M/10 NaCl. Changes in colonies occurred approximately at the seventh and fourteenth transfers in M/2 NaCl (S colonies became R; latter were granular on fourteenth and growth disappeared on fifteenth and sixteenth transfers), Ringer's (S colonies became R on eighth transfer and reverted to S upon fifteenth transfer), M/5 CaCl₂ (S colonies gave rise to R on fifth transfer; largest S possessed translucent thiosomes but subsequent R only few and small; growth disappeared tenth to sixteenth transfers when very R colonies appeared which were devoid of thiosomes), and M/10 CaCl₂ (S colonies with translucent or atypical thiosomes present

⁶ Asparagin 0.34 gram; ammonium lactate 1.0 gram; sodium chloride 0.50 gram; magnesium sulphate 0.02 gram; calcium chloride 0.01 gram; potassium phosphate 0.10 gram; glycerol 4.00 grams and agar solution 100.00 cc. For the NaCl broths, CaCl₂ was omitted and vice-versa. pH 7.8.

until fourth transfer when growth suddenly ceased; upon fifteenth transfer R colonies appeared). Atypical thiosomes appeared in both CaCl_2 concentrations only: in $\text{M}/5 \text{ CaCl}_2$ during the first to the ninth, and in $\text{M}/10 \text{ CaCl}_2$ during the first to fourteenth transfers.

With the smooth strain, 210S₂, in the salt broths, certain changes occurred in surface growth: about the sixth to seventh transfers in Ringer's (the usual hazy or thin type of pellicle absent), $\text{M}/5 \text{ CaCl}_2$ and $\text{M}/10 \text{ CaCl}_2$ (thin transparent pellicle disappeared upon sixth transfer to return twice upon irregular intervals); and again about the fourteenth transfer in $\text{M}/2 \text{ NaCl}$ (only appearance of a slight pellicle), $\text{M}/5 \text{ CaCl}_2$ and $\text{M}/10 \text{ CaCl}_2$ (return of thin, transparent pellicle). The ninth transfer showed a granular sediment in $\text{M}/2 \text{ NaCl}$, $\text{M}/10 \text{ NaCl}$ and Ringer's. Changes occurred in the colonies upon the following approximate transfers: fifth, Ringer's (S colonies became R upon fifth and sixth transfers and then reverted to S), $\text{M}/5 \text{ CaCl}_2$ (S colonies with irregular atypical thiosomes until fifth transfer when growth ceased; sixth transfer, small R colonies with few atypical thiosomes), and $\text{M}/10 \text{ CaCl}_2$ (S colonies with atypical thiosomes until fourth transfer, when R colonies devoid of thiosomes appeared; sixth transfer, reversion to S with atypical thiosomes); sixth, $\text{M}/10 \text{ NaCl}$; approximate eighth, $\text{M}/10 \text{ NaCl}$ (R to S), Ringer's (R to S upon seventh transfer), and $\text{M}/5 \text{ CaCl}_2$ (growth disappeared ninth to thirteenth transfers); and fourteenth, $\text{M}/5 \text{ CaCl}_2$ (small R colonies appeared) and $\text{M}/10 \text{ CaCl}_2$ (small R colonies appeared). Thiosomes appeared upon the following transfers: first, $\text{M}/2 \text{ NaCl}$; first to third and sixth to ninth, $\text{M}/5 \text{ NaCl}_2$; first to third and fifth to twelfth, $\text{M}/10 \text{ CaCl}_2$. It must be noted that the thiosomes were not typical, i.e., at no time did the opaque black type appear but only the translucent form (fig. 5 or fig. 20, submarginal zone). Little work has been done with impression preparations of these but they too are atypical.

DISCUSSION

It is evident from the literature which has accumulated that bacterial dissociation involves complex processes which are little understood and difficult of analysis and that bacterial protoplasm

has not the stability accredited to it by conceptions of the recent past, but responds in various ways to the inconstant physico-chemical changes taking place in its environment. During the course of studies on dissociation of certain paratyphoid bacilli, variant cells capable of giving rise to calcium sulphite crystals were found and herein are called thiosomes. These are referred to as thiosomes because indications to date point toward a type of variant cell associated with the sulphite crystals that probably goes to make up a colony-like structure. Their extremely close association with ordinary colonies makes attempts at cultivation most difficult but work is at present under way relative to this phase of the problem.

These variants grew from *S. Schottmülleri* filtrates (series I) in sealed glass ampoules which had become cloudy or opalescent, and also from the following sources: *S. Schottmülleri* in serial salt broth transfers which had not undergone filtration (series II); serial transfers of *E. coli*, *E. typhi*, *S. paratyphi*, *S. Schottmülleri*, *S. paratyphi*, type C (series III); *E. coli* isolated from a case of cervicitis (series IV); and atypical forms associated with *S. Schottmülleri*, single-cell R and S strains, in a synthetic medium (series V). In connection with the above, as well as with certain bacteriophage studies carried on during the past two years, daily transfers in broth have been made in a number of series of several paratyphoid cultures and in each instance certain characteristics appeared and disappeared with sufficient frequency to arouse interest relative to possible correlations of growth phenomena. Gram-stained smears were made daily from each broth and the definite changes in morphology at various intervals will be reported at a later date.

The media used have been fully described and the salts present have doubtless played an important rôle in these studies. Literature which deals with salt effects is extensive and since it has been admirably summarized and analyzed by Falk (1923), Buchanan and Fulmer (1930, vol. II) and others, it demands but few details here. As Fabian and Winslow (1929) point out, all electrolytes, studied by them, stimulate bacterial growth in low and inhibit it in higher concentrations. They found maximum stimulation to

occur with a "Na concentration of 0.10 M and pH of about 7.5;" thus, in the present work, M/10 NaCl, pH 7.4 to 7.8, served as a control as well as did the media to which no salts were added. Their investigation also indicated that a rapid "change from stimulation to inhibition" occurred when Na was increased. In this event, dissociation should be more marked in the M/1 NaCl and M/2 NaCl media; this was found to be true as shown culturally and also by a decrease in the number of motile cells. In a simple synthetic medium, Winslow and Dolloff (1928) showed that cations do not function qualitatively but only quantitatively and effects produced at given concentrations varied decidedly. In the present experiments, M/10 and M/5 CaCl₂ sometimes seemed to inhibit but at other times to accelerate growth to a greater or less extent; certainly, their effect upon different cultures varied, for example, in the scanty growth to which *S. paratyphi* gave rise. Just what the quantitative relationship is between NaCl and CaCl₂ in respect to dissociative changes would have to be ascertained by the use of a greater range of concentrations for each individual culture. Winslow and Haywood (1930) found by means of comparative tests, that stimulation and inhibition concentrations are about the same in distilled water, Dolloff's synthetic medium, or peptone solution. They obtained relative potency constants for eight cations and found that Ca is nine times more potent than Na. Bordet (1930) and Bordet and Renaux (1930) found that calcium constituted an important factor in the production of variants of the anthrax bacillus and they refer to changes induced in certain other bacteria relative to pigment production and morphologic variations when the calcium content of a medium was altered.

Although certain concentrations of NaCl exerted an effect upon the metabolic activities as manifested by variations in precipitation of sulphite crystals, it must be emphasized that other factors, doubtless much more deeply seated, are involved since thiosomes occurred repeatedly in media to which no salt had been added. There is much evidence to show, for example, some effect of S and R colony types on thiosomes or vice-versa; in the great majority of instances some S constituent or constituents seemed

to be necessary for thiosome occurrence (fig. 19) in fact, to date no extremely rough colony has been seen associated with thiosomes. In the synthetic medium, with R constituent or constituents probably present to a greater or less extent, there seemed to be created conditions favorable to the continuation of the formation of thiosomes. Not only did the S strain produce thiosomes intermittently in contrast to their constant presence with the R strain, but the S strain also was associated with thiosomes one transfer less in the $M/5$ $CaCl_2$ and two transfers less in the $M/10$ $CaCl_2$ than was the case with the R strain. Let it be recalled that the sulphur ions introduced in the form of magnesium sulphate were not found to be transformed into a sulphite so that atypical thiosomes only were formed.

Dissociative changes were probably accelerated by the use of a base for the salt bouillon and agar which was rich in nutritive constituents and retarded by the base in the synthetic medium which was poor in nutritive constituents. Since the synthetic medium permitted only a minimum growth, the colonies were always small and markedly transparent; S and R types were not clear cut and it is worthy of note, that under such apparently adverse conditions, thiosomes developed, atypical though they were. Adaptation of the two cultures to this medium probably would have been of little value, since in connection with other studies, this same medium was used for serial broth-to-broth and broth-to-agar transfers for over three months with little or no improvement in vigor of growth.

Observations indicate that thiosome formation is in some obscure way related to or influenced by bacteriophage. Thus far, sulphite crystals have not been found associated with areas on or near colonies where there is evidence of bacteriophage activity (figs. 17 and 22). Perhaps certain bacteriophage types could be found as suggested from the work of Burnet (1929) and Burnet and McKie (1930). Certainly on agar plates, at least, thiosomes in the great majority of instances are produced by comparatively young colony cells; they are usually associated with colonies on agar plates eighteen to twenty-four hours old, certainly not older than about forty-eight hours, with the exception, at times of the

atypical thiosomes on veal-infusion agar only. The latter appeared likewise with young cells on synthetic media and in the series of transfers reported there was no evidence of phage activity. There are undoubtedly factors which perhaps condition the metabolism of the colony to permit of the development of thiosomes, in fact, even permit at times the development of a definite type of thiosome such as the large elliptical ones.

Since the veal infusion and synthetic media provoked such variations in the response of the organisms to these highly nutritive and slightly nutritive bases, as well as to wide differences in NaCl and CaCl₂ concentrations, it is evident that general correlations relative to growth characteristics must be made with certain reservations. The two media must be dealt with separately. In veal infusion broths, with few exceptions, surface growth of some type was distinct approximately through first five transfers; about this time pellicles changed in character or sometimes disappeared. For the next five or six transfers growth characteristics of the initial three or four transfers generally prevailed. Thus, about the fifth transfer is suggestive of a first critical period and at about the 13th transfer a second such period was often evident.

These fluctuations in veal infusion broth surface growth characteristics can be correlated to a certain extent with motility. With the exceptions of the broths with the greater salt concentrations, a high percentage of motile cells was observed until about the first critical period when there was an abrupt decrease. This was also true about the twelfth to fourteenth transfers and in the interim, although there were variations, the trend was toward an increase in the percentage of motile cells. No motility tests were made in the serial broth transfers with the synthetic salt broths. Motility investigations have been of much interest since a motility-temperature relationship (results as yet unpublished)⁷ was found to exist among some of about 160 stock cultures of paratyphoid bacilli.⁸ The work herein reported as well as the literature that will be presented in a subsequent report relative

⁷ Jordan, E. O., and M. E. Caldwell.

⁸ Dr. E. O. Jordan's collection.

to this temperature-motility relationship, suggests that besides temperature or its influences, lack of motility may be due to an interval in a dissociative recurrent change when a type of cell predominates that either lacks flagella or possesses non-functional flagella.

The appearance or disappearance of thiosomes has been detailed and some sort of correlation between these bodies, colony variations, broth characteristics and the relative number of motile cells is suggested. In series II, the appearance of thiosomes when both surface growth and motility were inhibited by the high salt content of $M/1NaCl$, indicates an inherent potency or potencies which were not entirely suppressed by the environmental conditions imposed upon the organisms in these media.

From observations thus far made, there seems to be considerable indication that perhaps thiosomes are precursors of or in some way related to variant colonies which have been described by Hauduroy (1927), Vaudremecr (1921), Soule (1928), Hadley (1931a) and others. The first evidence of this arose in connection with the filtrate studies, series I. The scalloped translucent margin of thiosomes previously described (fig. 8), may have developed in response to the presence of $CaSO_3$ crystals but its occurrence might also be interpreted as a growth of variant cells in the colony-like structure that progressed after all available Ca^{++} and SO_3^{--} had united and therefore this newer growth lacked opacity due to the absence of sulphite crystals. What have been called atypical thiosomes were first observed in series II, but were studied in greater detail in series III (fig. 20, sub-marginal zone) and series V. Frequently associated with these on agar plates were transparent, extremely tiny to small colonies. *S. paratyphi* (series III), sixth daily broth transfer, after seventy-two hours incubation at $37^\circ C$. on $M/5 CaCl_2$ agar (fig. 15) gave rise to thiosomes that were of much interest primarily because of their variations in opacity. Those that either did not possess or had lost their greater degree of opaqueness or blackening showed differences in internal structures varying from extreme roughness to comparative smoothness; and of still greater interest were the innumerable tiny transparent colonies.

In keeping with the work which has shown that dissociation may be induced by salts and by differences in nutritive values of the medium and thereby give rise to sulphite crystals, it was not a surprise to find thiosomes associated with an atypical *E. coli* isolated from a case of cervicitis (series IV). It is not known whether the *E. coli* isolated was the etiological agent, but it is suggested that since this patient had received treatment three times a week for a month and had shown an improvement, the organism might have been affected not only by the condition of the host but also by the medicinal applications to which it had been subjected. Although this is a report of a single case, it aroused much interest not only because the thiosomes were worthy of considerable study, but also since the cells from direct smears and from young brain broth cultures, were similar to those seen upon first examination of sealed cloudy filtrates (series I) and which also developed into "normal" *E. coli* cells. It is planned to continue this work when material can be obtained from similar infections and secured prior to, as well as at intervals during, treatment.

It is suggested that the formation of some of the clinically important concretions in the animal body may be related to the ability of certain variant bacterial cells to precipitate calcium. In a review of the literature it is found that much uncertainty is expressed relative to the rôle which infection may play. Many citations that seem to have a distinct relation to the present work might be made but it is not within the scope of this paper to discuss the complex physico-chemical factors involved. In man, the most common calculi are those found in the biliary and urinary tracts and they are frequently associated with inflammations. While the nidus or centrum is frequently a mass of desquamated cells, fibrin, mucin or some type of foreign body, it has also often been found to be a clump of bacteria (Karsner, 1929), the colon and typhoid bacilli being most frequently incriminated, and bacilli have been found viable in gall stones for surprisingly long periods of time (Wells, 1925).

Concretions usually consist of a mixture of the materials present but are never composed of one substance in a pure form. Interest

here centers in the ever-present calcium salts of the bile pigments which are always found even though cholesterol is present to such an extent as to be referred to as a "pure" cholesterol stone (Wells, 1925). Besides the combination of calcium with the pigments, such as bilirubin and biliverdin, this element has been reported, for example, as a carbonate, phosphate, sulphate, and even oxalate (Wells, 1925). In the blood, proteins hold the calcium salts in solution or suspension in an unstable condition. Many hypotheses exist but an explanation of the causation of stones is still to be found. Lichtwitz (1910) has shown that when the gall bladder is inflamed calcium and magnesium are precipitated along with cholesterol, bilirubin and proteins. Since calcium is derived from the blood and is found to be present to the extent of about 0.1 gram per liter of serum (Wells, 1925), whereas in Difco-veal-infusion medium calcium was precipitated as a sulphite when only 0.0015 gram calcium per liter was found, it seems possible in the light of the present work that variant bacteria which may be present in such infections might find such an environment favorable for the precipitation of calcium with the consequent formation of calculi.

In support of this possibility, certain experimental biliary tract disturbances have been induced which resulted in changes in the calcium content (Wilkie, 1928; Andrews and Hrdina, 1931). Rous, McMaster and Drury (1924, I) induced lithiasis in dogs by intubation for the collection of sterile bile. They avoided stasis and state that infection played no part. Of their 22 experimental dogs, 35 per cent of the 14 dogs with stones were not used because of infections. No data were given as to the bacteria found in the infections. Even though the secretions were sterile in the dogs studied, is it not conceivable that in certain locations as the lower part of the cannula "coated with organic debris and in the midst of . . . calculi," there might have been bacterial variants not readily cultivable, not responsible for a frank inflammation and yet capable of playing some part in the precipitation of calcium salts normally present? Stone formation seemed to be definitely associated with the presence of organic debris and the authors statement that "calculi never developed on the surface

of the material but always within it as if determined by some special condition there" suggests in light of the present investigation some type of bacterial activity. Of interest in this and especially in a second paper (Rous, Drury and McMaster, 1924, II), is the description of the nuclei of stones. Many types of calculi were found to contain minute spherical nuclei which in their photographs, plate 17, figures 9 and 10, appear most similar to thiosomes. Since these nuclei effervesced but slightly when treated with dilute HCl they were thought to contain only a small amount of carbonate; however, the present work would suggest that this reaction may have been due to the liberation of SO_2 . Rous, et al. (1924, II) state that "whole crops of stones were sometimes present side by side. It was as if at some critical period in bile collection bilirubinate had come out of solution providing here and there centers favorable to subsequent layering with another material. . . . The question arises as to the possibility of a correlation between the "crops" and the often sudden appearance and disappearance of thiosomes in the present investigations. When CaCl_2 was injected intravenously into the dogs a "shower" of nuclei sometimes occurred. This might be correlated with the finding of the enormous numbers of soot-like thiosomes when CaCl_2 was purposefully added to media. The authors note that in bile from human gall bladders with concretions, calcium carbonate spheruliths were found and thought to act as nuclei for the formation of secondary stones.

At this time, no attempt can be made to cite the results of other investigations which indicate the possibility that bacterial dissociants may have been involved in the formation of calculi. Under various pathological conditions there are records of the inability to culture bacteria when observed microscopically, of irregularly stained cells, of loss of viability under certain conditions, morphological, colonial and cultural variations together with lack of motility of certain bacilli all of which are suggestive of the above hypothesis (Mackey, 1931; Martin, 1929; Nauss, Lake and Torrey, 1931; Judd, 1927; Wagner, 1922; Branch, 1929; Nagai and Watanabe, 1927; Olivieri, 1929; and Kelly and Dible, 1930).

In the growth and metabolism of bacterial cells in general sulphur is apparently an essential element (Buchanan and Fulmer, 1930, II). Most cells assimilate this in the form of sulphates although other compounds are sometimes utilized. Much of the work reported involves studies of H_2S production by bacteria, as well as some other sulphur compounds, but few references have been found relative to the formation of sulphites. Since normal sulphites slowly add on oxygen, and sulphate enters as an impurity this may have obscured its detection at various times; also, the identification of a sulphite might have been possible had microchemical technique been taken advantage of in bacteriological work to a greater extent than the literature indicates. Cystine is present in almost all bacteriological peptones (Buchanan and Fulmer, 1930, III) and is doubtless the source of various sulphur compounds. Tilley (1923b) stated that "commercial peptones have been shown to contain unoxidized, partly oxidized and oxidized sulphur in varying proportions" and found variations in the amount of hydrogen sulphite produced by bacteria. This paper, as well as a second one (1923a), also suggests the possibility of the presence of variant bacterial cells. Almy and James (1926) in their studies concluded that decomposable sulphur compounds in peptones were promptly utilized and H_2S formed during the stage of rapid multiplication of bacteria. In the present investigation, this early utilization may be correlated with the usual appearance of calcium sulphite crystals in association with relatively young cells. Almy and James (1926) also found variations in the amount of H_2S produced from five brands of peptones commonly used in laboratories; it would be of interest to know how "Bacto-" peptone was valued. Their statement that the rate of H_2S formation was not uniform with *S. aertrycke* or *Proteus vulgaris* suggests that certain dissociative factors may have been active. This variation in rate, should it be found to be applicable to sulphite production, might account for the fluctuations in the number and rather decided differences in appearance that thiosomes exhibited from time to time. In other experiments, when proteolysis was inhibited, more or less, by the addition of glucose, a principle thoroughly investigated

by Kendall and his colleagues (1912), calcium sulphite was not precipitated which indicates the retardation of N metabolism with its consequent prevention of the utilization of sulphur-containing derivatives of protein by the bacteria. McLeod (1928) points out that bacteria capable of forming H_2S probably do so more readily if cystine is added to the medium and yet, there is evidence to the contrary; that some of the coliform bacteria have this ability is recognized by many workers.

By more or less controlling the constituents in media responsible for the formation of $CaSO_3$ crystals, something of an insight into the activities of certain members of the colon-typhoid group may be gained but let it be emphasized that this does not necessarily mean that the same abilities are manifest in the very different environment of an animal body. However, there are several well known conditions that might show some correlation. Patients with cystinuria frequently develop concretions in the kidneys or bladder and sulphates and other neutral sulphur compounds are present in the urine (Wells, 1925). Under such conditions may not the formation of these calculi be incited in part, perhaps, by bacterial activities? Since concretions are often associated with abnormal intestinal putrefaction of protein and since the amounts of ethereal sulphates in urine indicate the extent of this intestinal putrefaction (MacLeod, 1922) it seems possible that certain variant bacterial cells in the urinary bladder might form sulphite crystals; this also might occur in the biliary tract provided ethereal sulphates which are probably formed in the liver (MacLeod, 1922), also find their way into bile. In the gall bladder, therefore, the nuclei already referred to in connection with the work of Rous, et al. (1924) may have had their inception in the form of calcium sulphite crystals as a nidus upon which calcium salts or cholesterol may have been deposited. In the biliary duct system when inflammation leads to a lessened motility with its consequent greater accumulation of organic debris than is normal, then such an environment probably becomes more favorable for bacterial activities. These activities are doubtless little understood and are taking place where perhaps sulphur containing derivatives are not only abnormally high, but furthermore

in an environment where tissues absorb certain sulphur ions at times with great difficulty (Hawk and Bergeim, 1927). The results of this investigation indicate that with certain members of the colon-typhoid group the metabolism of calcium and sulphur is modified by dissociative phenomena.

SUMMARY

This paper deals with what are thought to be variant bacterial cells which have the ability to precipitate calcium sulphite in a suitable environment. Since these variant cells seem to give rise to a daughter-colony-like structure they are termed thiosomes. Two types of the latter are described.

Data are presented relative to the finding of thiosomes from the following sources: sealed filtrates of *S. Schottmülleri* (series I); serial salt broth cultures of *S. Schottmülleri* used in certain bacteriophage studies (series II); serial salt broth cultures of *E. coli*, *E. typhi*, *S. paratyphi*, *S. Schottmülleri* and *S. paratyphi*, type C (series III); *E. coli* isolated from a case of cervicitis (series IV); and atypical thiosomes associated with *S. Schottmülleri* in a synthetic medium (series V).

Evidence that the fundamental structure of a thiosome is colony-like appears as follows: (1) Atypical thiosomes resemble the opaque thiosomes in size, in structure, in position on the colonies and in their irregular occurrence. With polarized light atypical thiosomes do not appear to be of a crystalline nature. (2) Lack of any definite pattern of sulphite crystals when numerous and, when few in number, their formation on the initial streak of the plate only, suggest the presence of scattered variant cells. (3) Intermittent occurrence of thiosomes during serial transfers in the same lots of media to which either no salts or NaCl had been added; also, variations in numbers of thiosomes in the control CaCl₂ media. (4) The association of thiosomes with S and R colony types. (5) The development on the part of some thiosomes of scalloped margins which have either lost their blackness or are less opaque. (6) By application of suitable reagents, CaSO₃ crystals may be dissolved and leave a colony-like structure devoid of any blackness.

Conditions which promote dissociation seem to be conducive to the development of thiosomes, such as age, salts, rapid transfers in broth, composition of medium, and probable activity or activities of bacteriophage. These thiosomes were found, however, in media which contained the usual NaCl concentration, about $m/10$ NaCl, as well as in media to which no salt had been added, which indicates an inherent potency or potencies which, within certain limitations, may not be under the control of environmental conditions.

In serial broth transfers, recurrent changes occurred relative to surface growth, turbidity, sediment and motility. Agar plates streaked from these serial broth cultures indicated that, in the latter, thiosomes were concerned in these changes together with the variations in S, Sr, and Rs and R type colony characteristics.

The possibility that certain variant bacterial cells may have the ability to precipitate calcium and hence be of some significance in the formation of certain concretions in the animal body is discussed.

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PLATES

PLATE 1

FIG. 1. Thiosomes associated with *S. Schottmülleri* colonies, from sealed m/10 NaCl broth filtrate; grown on m/10 NaCl agar; twenty-four hours 37°C. $\times 15$.

FIG. 2. Thiosome, Gram stain, impression preparation. Note granules within capsule-like structure. $\times 1540$.

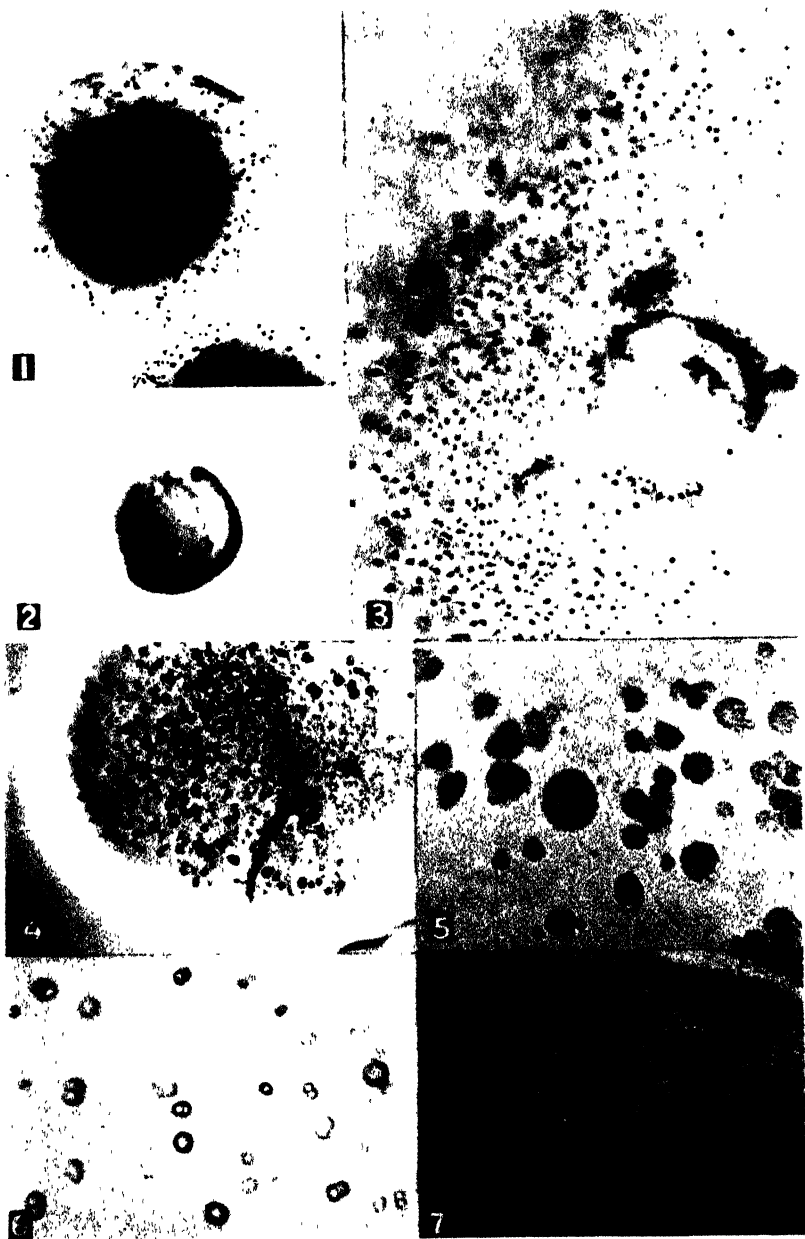
FIG. 3. Ruptured thiosome, Gram stain, impression preparation. Note capsule-like structure from which embedded granules have escaped. $\times 1540$.

FIG. 4. *S. Schottmülleri*, Bacto-H₂O agar; forty-eight hours 37°C. $\times 20$. Typical (opaque) and atypical or translucent thiosomes. Note greater number of atypical thiosomes within segment of colony.

FIG. 5. *S. Schottmülleri*, fresh veal infusion m/10 NaCl agar. $\times 100$. Atypical thiosomes superimposed upon a colony.

FIG. 6. Background of this photograph is surface of *S. Schottmülleri* colony. Fresh veal infusion m/10 NaCl agar. $\times 100$. This colony was devoid of any type of thiosome; upon addition of $\text{Ca}(\text{C}_2\text{H}_3\text{O}_4)_2$, CaSO_4 crystals were produced.

FIG. 7. *S. Schottmülleri*, Bacto-H₂O agar, fourth transfer. $\times 100$. Opaque thiosomes treated with H_2SO_4 showed presence of calcium in the formation of these typical CaSO_4 crystals.



(Mary E. Caldwell: Dissociation of Paratyphoid Bacilli)

PLATE 2

FIG. 8. *S. Schottmulleri*, from sealed m/10 NaCl broth filtrate. Growth on m/10 NaCl agar after 10 daily transfers in m/10 NaCl broth; twenty-four hours 37°C., plus fourteen days 8°C. $\times 75$. Series I.

FIG. 9. *S. Schottmulleri*. Growth on m/10 NaCl agar after 10 daily transfers in m/10 NaCl broth; eighteen hours 37°C. $\times 75$. Series II.

FIG. 10. *S. Schottmulleri*. Growth on m/10 NaCl agar after 10 daily transfers in m/10 CaCl₂ broth; eighteen hours 37°C. $\times 75$. Series II.

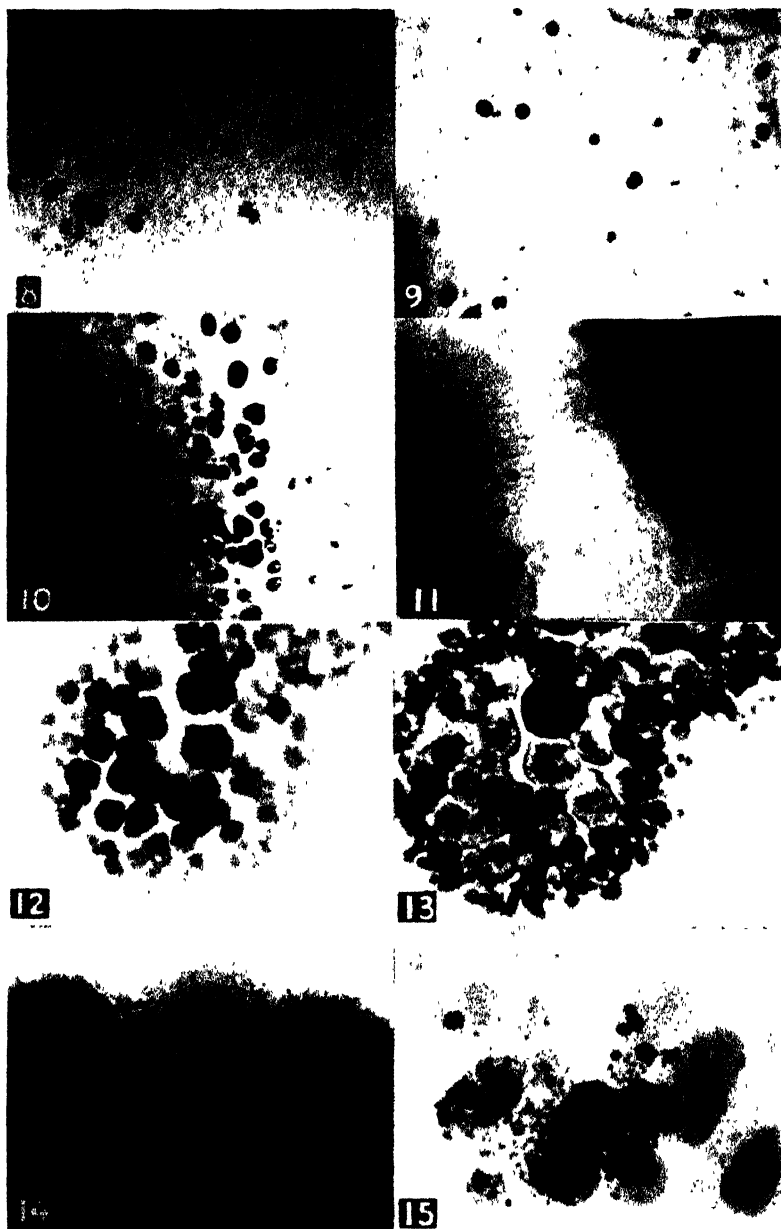
FIG. 11. *E. coli*, m/10 NaCl agar, sixth transfer; seventy-two hours 37°C. $\times 40$.

FIG. 12. *E. coli*, m/5 CaCl₂ agar, sixth transfer; seventy-two hours 37°C. $\times 40$.

FIG. 13. *E. coli*, m/5 CaCl₂ agar, sixth transfer, twelve days 37°C. $\times 40$. Same field as figure 12.

FIG. 14. *E. typhi*, m/5 CaCl₂ agar, sixth transfer; seventy-two hours 37°C. $\times 40$.

FIG. 15. *S. paratyphi*, m/5 CaCl₂ agar, sixth transfer, seventy-two hours 37°C. $\times 40$.



(Mary E. Caldwell: Dissociation of Paratyphoid Bacilli)

PLATE 3

FIG. 16 *S. paratyphi*, type C, m/5 CaCl_2 agar, sixth transfer; seventy-two hours 37°C. $\times 40$

FIG. 17 *E. coli*, m/5 CaCl_2 agar, seventh transfer; forty-eight hours 37°C $\times 40$.

FIG. 18. *E. coli*, m/5 CaCl_2 agar, seventh transfer; forty-eight hours 37°C $\times 40$.

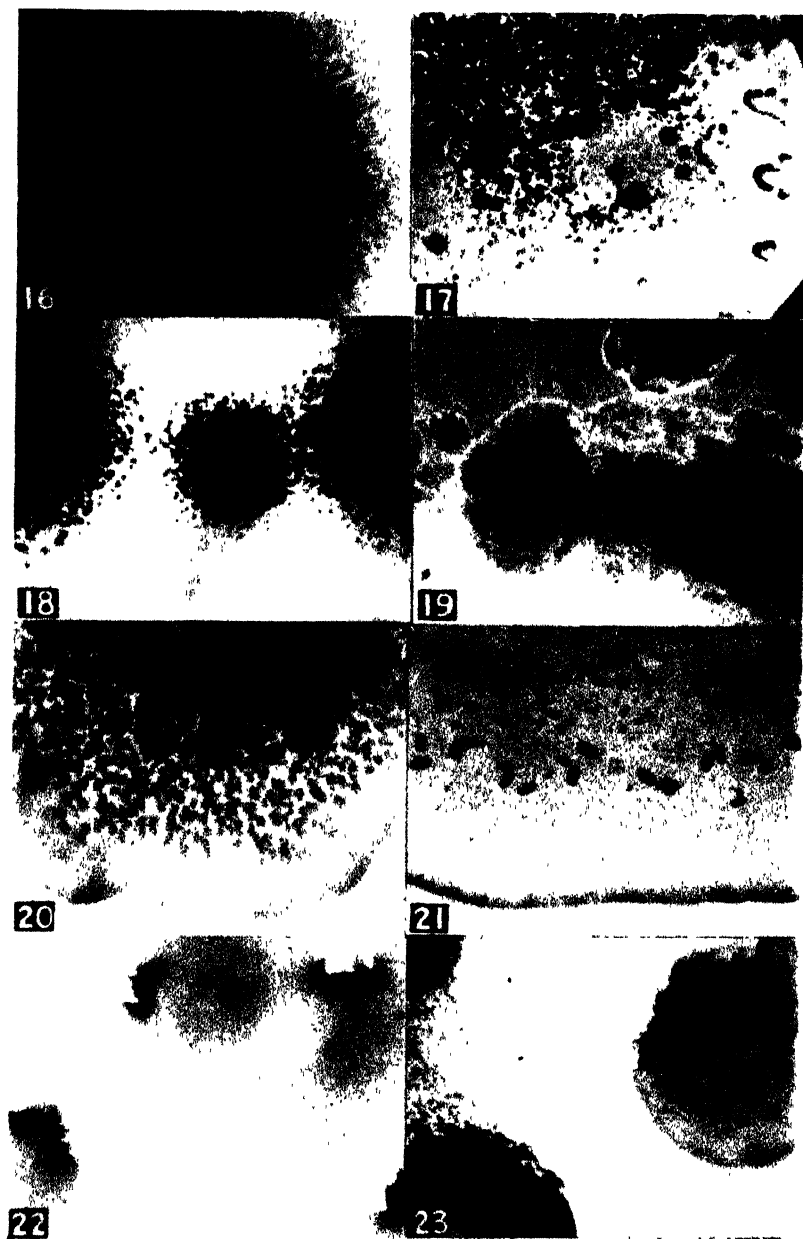
FIG. 19 *E. coli*, m/10 NaCl agar, eighth transfer; twenty-four hours 37°C $\times 40$.

FIG. 20 *E. coli*, m/5 CaCl_2 agar, eleventh transfer; seven days 37°C $\times 40$

FIG. 21. *S. Schottmulleri*, m/10 NaCl agar, thirteenth transfer; forty-eight hours 37°C. $\times 40$

FIG. 22 *S. Schottmulleri*, m/10 NaCl agar, thirteenth transfer; forty-eight hours 37°C $\times 40$

FIG. 23 *E. typhi*, m/5 CaCl_2 agar, fifteenth transfer; forty-eight hours 37°C $\times 40$.



A BACTERIOPHAGE FOR *CL. TETANI*

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Bacteriophages have been obtained for so many aerobic organisms, both spore-formers and non-spore-formers, as well as for the microaerophile, *Cl. tertium*, that it seems reasonable to believe that the phenomenon is a general one applying to all species of bacteria, at least, and that, if desired, the presence of lytic principles for them all can be demonstrated. As yet, however, the literature on the subject has contained no report of a bacteriophage active against any of the strict anaerobes, and for this reason attempts were made to obtain a lytic agent for *Cl. tetani*.

Two-hundred-cubic-centimeter volumes of crude sewage and double strength infusion broth were mixed and inoculated with 0.5 cc. amounts of forty-eight-hour cultures of the following strains of *Cl. tetani* obtained from the American Type Culture Collection—Nos. 444, 445, 457, 3598, and 4260. After twenty-four hours' incubation at 37° in vacuo this mixed culture was filtered through a Chamberland candle, and 0.5-cc. amounts of the filtrate added to tubes of broth. Each tube received, as well, a 0.1-cc. inoculum of an eighteen-hour broth culture of one of the strains, and duplicate cultures were made without filtrate to serve as controls. Cysteine HCl was routinely added to all media in a concentration of 0.1 per cent in order to ensure vigorous growth overnight. After several serial passages of the filtrates against the homologous strains a lytic principle was obtained active against No. 444 and to a lesser degree against No. 457. The other strains proved to be resistant. Subsequently, several passages were made of filtrates from the resistant strains against No. 444 in order to determine whether they had been sources of the bacteriophage. The results were negative.

At this place it may be well to emphasize the desirability of using an adequate number of different strains when attempting to isolate a lytic principle for any species of organism for which none has been found, for in different groups the percentage of readily susceptible strains may vary greatly.

This bacteriophage, in its production of plaques, its transmissibility in series, its filterability, and its resistance to heat, behaves as do others. There is apparently no lysis under aerobic conditions. Moreover, by appropriate contact between culture and bacteriophage the former can be made resistant and lysogenic, and cultures derived from heated spores of such a lysogenic culture contain bacteriophage, as is the case with other spore-forming bacteria (Cowles, 1931).

In order to determine whether or not the presence of bacteriophage in a culture over a long period of time would alter the metabolism in such a way as to influence the production of either of the two tetanus toxins, tetanolysin or tetanospasmin, titrations were made at intervals over a period of a year and a half, but no significant differences were noticed in either case. Presumably, therefore, the presence of bacteriophage cannot be held responsible for the poor toxin production characteristic of some strains of *Cl. tetani*.

SUMMARY

A typical bacteriophage has been obtained for *Cl. tetani*. It has no effect, apparently, on toxin production.

REFERENCE

- COWLES, P. B. 1931 The recovery of bacteriophage from filtrates derived from heated spore suspensions. Jour. Bact., **23**, 119.

BACTERIAL MOTILITY¹

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Motility is universally recognized as a significant biological character of certain bacteria, and is used as a fundamental basis of classification in many taxonomic systems. Significance is often attached to motility as an identifying character in the differentiation of species, and independent species have sometimes been established on the basis of lack or possession of motility. Relatively little attention seems to have been paid to variability in motility and the factors that influence it. Discordant statements and opinions about the presence or absence of motility in different species are, however, not difficult to find. Although the overwhelming weight of opinion today is that dysentery bacilli are non-motile (Lentz and Prigge, 1931; Gardner, 1929), both Shiga and Flexner originally described these organisms as motile and several experienced observers have since reported that motility either occurs spontaneously in certain strains or may be induced by the application of special methods.² As regards other groups of bacteria, such as the so-called Morgan bacilli, quite divergent statements as to the presence or absence of motility are made. It is well known also that non-motile strains of predominantly motile species sometimes occur; Smith and Reagh's classical observations (1903) on the distinction between somatic and flagellar agglutinins were based on the discovery of a strain of the hog-cholera bacillus which was non-motile but in all other respects appeared to be identical with the motile type. The effect of environmental factors on motility seems to have been little studied.

¹ The present investigation was aided by a grant to the University of Chicago from the Rockefeller Foundation.

² It has been found, for example (Colquhoun, D. B., and Kirkpatrick, J., 1932), that many organisms, including a dysentery strain, that are non-motile in ordinary fluid or solid media develop motility when grown on semisolid medium.

Occasional references to the effect of temperature upon the motility of bacteria may be found scattered through the literature. Thus Migula (1897), in discussing the effect of slight environmental changes upon motility, writes: "Der *Bacillus prodigiosus* ist beispielsweise gewöhnlich unbeweglich, er wird aber sehr lebhaft beweglich, wenn er bei Blutwärme gezogen wird." Matzschita (1901) noted that certain strains of *B. coli* and *B. typhosus* evinced more lively motility at 20°C. than at 37°C. The bacteria of the intestinal group appear to be most often cited in observations of this sort. Mironesco (1899) studied a typhoid-like bacillus which was absolutely non-motile and devoid of flagella at 38°C., but actively motile at 23°C. Kossel and Overbech (1902) briefly noted that three strains of bacilli from pseudotuberculosis in guinea pigs were motile when grown at room temperature, but when left at 37° showed no motility. Nicolle and Trenel (1902) isolated from a guinea pig a typhoid-like bacillus that was non-motile at 25° to 35°C., but became motile when grown at 18° to 20°C. De'Rossi (1904) observed a coli-like bacillus which was actively motile when grown at 15°C. but at 37° almost completely non-motile. Neustadtl (1917), in studying motility in coli-like bacteria, found that some strains were motile at one temperature, non-motile at another; in most instances motility was most pronounced at 37°, but in three strains the motility was greater at 18° than at 37°. Braun and Löwenstein (1923-4) observed a dysentery-like bacillus, named by them *B. inconstans*, which was non-motile at 37°C., motile at 22°. Braun and Weil (1928) noted the occurrence in stools of bacilli culturally similar to the dysentery bacillus, but which were motile when grown at 22°C. although non-motile at 37°.

In spite of these scattered observations, the effect of temperature on motility does not seem to have been generally recognized by bacteriologists, and the usual "determination of motility" for diagnostic purposes is based on examination at one temperature only.³

³ See for example Committee on Bacteriological Technic of the Society of American Bacteriologists: Manual of Methods for Pure Culture Study of Bacteria, Leaflet V, Fourth Edition, 1930, V₃₃-4.

METHODS

For examination by the usual hanging drop method the cultures of about 40 strains of *Salmonella Schottmulleri* were grown in standard veal-infusion broth at 22° and 37°C., fresh young cultures (twenty to twenty-four hours) being always employed.

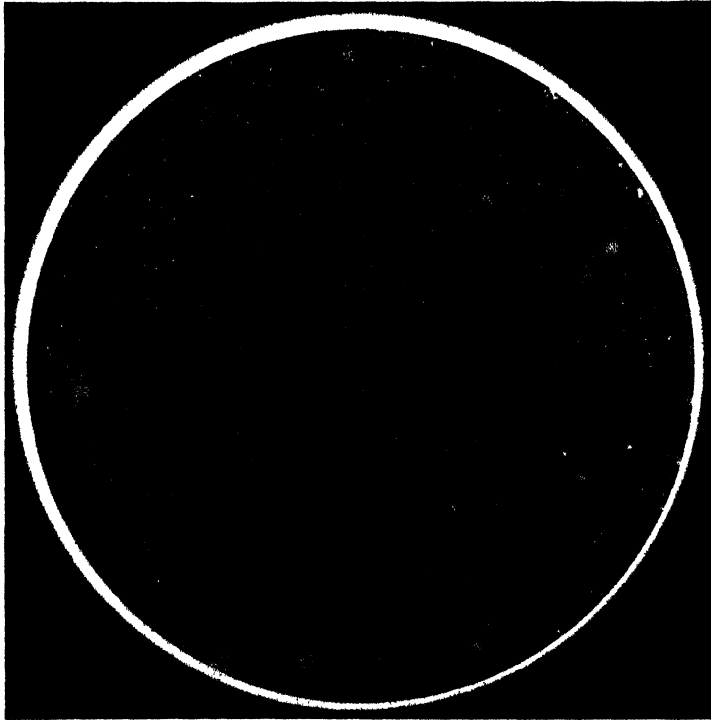


FIG. 1. *S. SCHOTTMULLERI* 209 INCUBATED 37°C. TWENTY HOURS.
NON-MOTILE COLONY TYPE

The plate shows approximately 219 colonies. There are 21 surface colonies and 10 bottom colonies.

The percentage of motile cells may be roughly approximated by drawing a ring with a wax pencil on the lower lens of the eyepiece and inspecting several fields of the drop within the ringed area. The hanging drop method has its limitations: the observer may at times find it difficult to detect the presence of a few non-motile

organisms in a field of actively moving ones and it is impossible to determine whether cells not showing motility are alive or dead. During the division period some cells have been observed to become non-motile. In an actively motile culture, an apparently non-motile bacillus with a deep constriction was watched by one

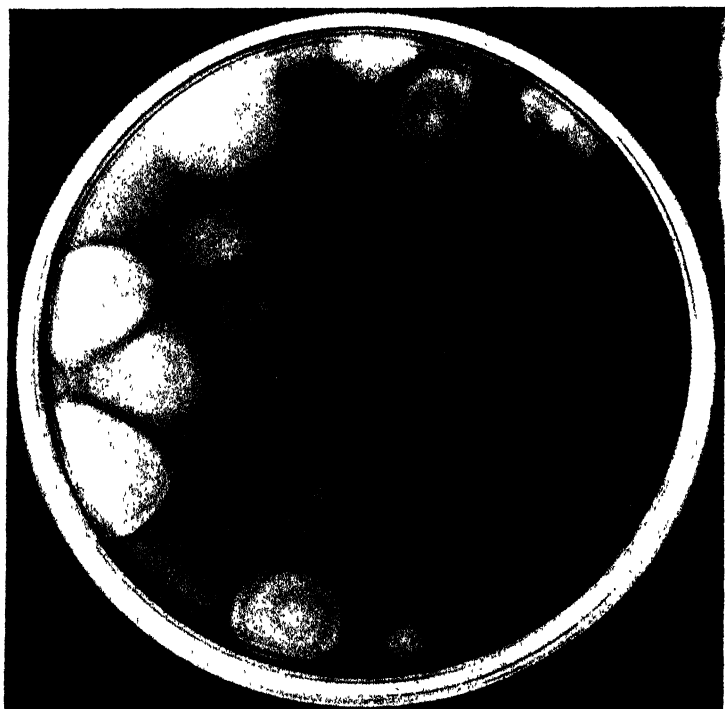


FIG. 2 *S. SCHOTTMÜLLERI* 379 INCUBATED 37 C. TWENTY HOURS MOTILE COLONY TYPE

of us (M. C.) for twenty minutes; soon after division was complete, both daughter cells exhibited motility.

A semi-solid medium, similar to that used by Hiss (1902) and by Li (1929), has also been employed in studying the property of motility. To a base of standard veal-infusion broth (pH 7.2 to 7.3) 0.3 per cent agar and 4 per cent gelatin are added for 22°C. cultures, and 0.5 per cent agar and 8 per cent gelatin for 37°C.

cultures. A uniform and carefully adjusted consistency is important since a medium that is too stiff does not permit adequate differentiation between the motile and non-motile colonies and an over-soft medium leads to blurring of the colony outlines. Overcrowding of the plate must be avoided. As pointed out by Li, the significant colonies on the poured plate are those embedded in the substance of the medium; the colonies on the surface and those at the bottom next the glass are spreading and often do not show any characteristic difference. Figure 1 shows the non-motile, and figure 2 the motile colony type. An interesting phenomenon, which might lead to confusion, is the change in colony type on a semi-solid medium when plates seeded with an organism non-motile at 37° but motile at 22° (see group 4) are first incubated at 37°C. and then allowed to stand at 22°; the non-motile type of colony changes to the motile. One disadvantage of the semi-solid medium is that if only a small proportion of motile cells are present in the culture they may be entirely missed unless many plates are made.

Forty-one strains of *Salmonella Schottmülleri*, culturally and agglutinatively identical and all of the smooth type,⁴ were examined in broth and in semi-solid medium: thirty-four of these strains were motile in approximately the same degree both at 22° and 37°, 4 strains were non-motile at both temperatures, 2 strains produced both motile and non-motile colony types on semi-solid agar and showed motile and non-motile cells in hanging drop preparations. One strain showed 100 per cent motile cells in broth cultures grown at 22°C. but in those at 37°C. usually none or at most 2 per cent; with this strain no colonies definitely of the motile type could be observed on the plates of semi-solid medium incubated at 37°. Three single cell strains derived from the parent strain gave similar results.

The four groups—all smooth strains—may be more particularly described:

1. *Highly motile both at 22° and 37°C. (34 strains).* Observations in hanging drop indicated that from 95 to 100 per cent of

⁴ The rough type is more irregular in behavior and for simplification is not dealt with in this paper.

the cells were motile. Marked gradations in colony size on the semi-solid medium at 22° (forty-two hours) were observed ranging from 3 mm. in diameter to 26 mm.; two strains gave colonies only about 1 mm. in diameter although both showed predominantly motile cells in hanging drop. Motility, therefore, cannot be surely adjudged by mere inspection of colonies on semi-solid plates. There is no definite relation between the size of the colony on semi-solid medium and the degree of motility observed with the microscope: those strains producing colonies 3 mm. or less are, to the eye, as actively motile as those producing colonies of 15 to 20 mm. In a general way, however, the highest proportion of motile cells, as observed by the microscope, occurred in those strains giving the largest colonies: at 22°, 4 strains were so actively motile that not a single non-motile cell could be observed in the hanging drop and all of these produced colonies 20 mm. in diameter or larger; at 37°, 6 strains reckoned as "100 per cent motile" in the hanging drop gave colonies 15 to 25 mm. in diameter. The method of comparison is a rough one and great variations are observed. The degree of motility, as observed in hanging drop and as indicated by colony size, was practically the same at 22°C. and at 37°C. for all these 30 strains.

2. *Non-motile both at 22° and 37°C. (4 strains).* These 4 strains showed no motility in broth either at 22° or 37° and no motile colonies on semi-solid medium.

3. *Predominantly non-motile, but some motile cells usually observed.* Two strains (nos. 288 and 292) showed a few motile cells in broth culture and occasional motile colonies on semi-solid medium, but the majority of the cells were non-motile. In one series, one colony out of 170 on semi-solid medium was of the motile type. The predominance of non-motile cells was manifest both at 22° and 37°C.

4. *Predominantly motile at 22°C., predominantly non-motile at 37°C.* One strain (210),⁵ although motile at 22°, has consistently remained almost completely non-motile when grown at 37°, an

⁵ This strain was first isolated in 1916 from the blood of a patient in San Antonio, Texas. Four single cell strains derived from the parent strain agree in their temperature-motility relations.

observation extending over two years. It is typically smooth and the colonies on ordinary agar are not different in appearance from those of the strains in group 1 (motile at both temperatures). Cultural and agglutination reactions are characteristic of the Schottmüller group.

FLAGELLAR STAINS

The results obtained by observations of the hanging drop and of the type of colony formation in semi-solid medium have been supplemented by counts of flagellated and non-flagellated cells. The method used for staining flagella is that described by Safford and Fleisher (1931) which, with slight modification, has given excellent results.

Method. Organisms from the water of condensation of twenty-hour veal infusion agar slants were transferred to a small volume, 1 to 2 cc., of sterile distilled water, until a barely perceptible turbidity was produced. The aqueous emulsion was then incubated for thirty to sixty minutes. After incubation the suspension was placed on clean slides and dried in the air.

When dry the preparation was covered with freshly prepared fixative, heated to steaming and the fixative allowed to act for one to two minutes. The fixative⁶ was then washed off with distilled water and the slide allowed to dry, after which the slide was covered with Fontana spirochaete stain⁷ and heated to steaming. The stain was allowed to act for one to two minutes, and the slide then washed with distilled water and dried.

Twenty-hour cultures were uniformly used in making the flagellar stains. Only those slides or sections of slides were studied which were well stained and showed an even distribution of organisms.

For control of the staining technique, a known motile organism was treated on each slide.

⁶ The fixative was prepared as follows: 100 cc. one-fourth saturated (aqueous) solution of picric acid (temperature of saturation, 22°C.); 5 grams tannic acid; 7.5 grams ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$).

⁷ The Fontana spirochaete stain must be freshly prepared as follows: To 25 cc. of 2 per cent AgNO_3 add ammonium hydroxide, CP, diluted 1 to 3 till the precipitate which forms redissolves. Then add more AgNO_3 till faint turbidity results. A clear solution is useless.

TABLE 1
Flagella stains

STRAIN	22°C.					37°C.				
	Number flagellated	Number non-flagellated	Total	Per cent flagellated	Per cent non-flagellated	Number flagellated	Number non-flagellated	Total	Per cent flagellated	Per cent non-flagellated
Group 1										
12	1,112	152	1,264	87.97	12.03	968	86	1,054	91.8	8.2
149	1,170	131	1,301	89.9	10.1	1,405	438	1,843	76.2	23.8
169	1,054	156	1,210	87.1	12.9	626	60	686	91.3	8.7
211	963	51	1,014	95.0	5.0	436	89	525	83.05	16.95
303	1,130	78	1,208	93.5	6.5	1,034	82	1,116	92.7	7.3
308	966	140	1,106	87.3	12.7	576	127	703	81.9	18.1
559	1,172	98	1,270	92.3	7.7	1,068	88	1,156	92.4	7.6
652	1,194	104	1,298	92.0	8.0	1,071	132	1,203	89.03	10.97
684	1,014	89	1,103	91.9	8.1	1,098	70	1,168	94.0	6.0
707	912	80	992	91.9	8.1	1,040	84	1,124	92.5	7.5
Group 2										
175	0	1,060	1,060	0	100	0	1,000	1,000	0	100
209	0	1,300	1,300	0	100	0	4,228 (1)*	4,229	0	100
209S	0	1,004 (5)*	1,009	0	100	0	1,032	1,032	0	100
221	0	1,008 (6)*	1,014	0	100	0	2,959	2,959	0	100
316	0	1,349	1,349	0	100	0	2,000 (3)*	2,003	0	100
Group 3										
288	80	976	1,056	7.6	92.4	132	3,066	3,198	4.1	95.9
292†	186	3,456	3,642	5.1	94.9	63	954	1,017	6.2	93.8
Group 4										
210	772	465	1,237	62.4	37.6	56	1,858	1,914	2.9	97.1
210-S ₂	723	283	1,006	71.9	28.1	0	2,500	2,500	0	100
						210	1,509	1,719	12.2†	87.8

* Probably all non-flagellated organisms. There was, however, a slight haze, perhaps due to precipitated stain, around a few bacteria, the number indicated in parentheses, so that it was impossible to be perfectly certain that flagella were not present.

† The figures for this strain are subject to error since the flagellated organisms were not evenly distributed over the field, but occurred in clumps. There was a decided predominance of non-flagellated organisms.

‡ This figure is unusually high. It represents that portion of the slide which showed the maximum number of flagellated organisms. Many fields showed only non-flagellated forms. 210-S₂ is like 292 in that the flagellated organisms were limited to certain parts of the slide.

The results of counting flagellated and non-flagellated cells are shown in table 1. While, on the whole, they are in harmony with the observations on hanging drop and semi-solid medium, they bring out several additional points of great interest. It appears that non-flagellated cells occur in cultures of all motile strains in varying proportions. On the other hand, in some instances an occasional flagellated cell may be observed among a large number of non-flagellated cells (group 3). The temperature at which the culture is grown has a marked effect upon the proportion of flagellated cells in one strain (group 4).

SUMMARY AND DISCUSSION

Within a given bacterial "species" carefully studied and delimited by cultural and agglutination absorption tests (E. O. J.) different degrees of "motility" exist. Some strains, under ordinary conditions of cultivation, consist practically entirely of motile cells, other strains show no motility in hanging drop or in semi-solid medium and no flagellated cells can be found in stained films. Non-motile strains of species predominantly motile are probably more common among bacteria than generally supposed. Four out of about 41 strains of *S. Schottmülleri* examined have remained consistently non-motile over a period of several years.

The percentage of motile cells in cultures of a given strain appears usually to be quite constant but may occasionally show considerable variation, as in strain 288, which in May, 1930 was composed of motile and non-motile cells in about the same proportion and again in April, 1931 showed a similar mixture. Examination in January, February and May, 1932, however, in both the hanging drop and semi-solid medium showed a very small percentage of motile cells (2 to 3 per cent) and this was confirmed by the relatively small proportion of flagellated cells seen in stained preparations. Throughout this period, the strain remained smooth so far as could be determined by repeated examination of broth cultures and of colonies on agar plates.

In one instance (strain 210 and single-cell derivatives) a definite temperature relation was found to exist over a three-year period of observation. Although little or no motility was manifested

by the cells from cultures of this organism grown at 37°C., two-thirds or more of the cells grown at 22° were motile. This relation was further confirmed by flagella stains which showed 60 to 70 per cent of flagellated cells in 22°C. cultures and a much smaller number in 37° cultures. In one series of slides of 37° cultures not a single flagellated cell was found in 2500 examined (table 1).

The following conclusions appear warranted:

In general, the proportion of motile cells in a given strain remains fairly constant when particular attention is given to conditions of growth and testing. Certain strains, however, may show considerable variation in the proportion of motile cells even when cultures are maintained and handled in as uniform a manner as possible.

Environmental conditions affect motility in some strains. A strain actively motile when grown at 22°C. may be practically non-motile when grown at 37°; the motility of other strains remains apparently uninfluenced by changes in temperature.

Motility is not a character that can be used for exact delimitation of species or varieties of bacteria. Standard cultural characters and biochemical reactions do not appear to be correlated with the presence or absence of motility.

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THE DIFFERENTIATION OF LIVING FROM DEAD BACTERIA BY STAINING REACTIONS

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Apparently the first efforts to differentiate living from dead bacteria by staining reactions were made in connection with Metchnikoff's theory of phagocytosis which was combated in its early phases by German authorities on the ground that phagocytes were only scavengers which, although capable of ingesting dead bacteria, were not endowed with the ability to include and destroy living microorganisms. Metchnikoff himself showed as early as 1887 that anthrax bacilli when ingested by phagocytes gradually acquired, coincident with their disintegration, the property of staining with Bismarck brown (Vesuvine) whereas living bacteria outside the cells and in the initial stages within the cells failed to take this dyestuff.

In 1895 both Mesnil and Bordet showed that the natural affinity for methylene blue of living bacteria, outside the phagocytes and, in their initial stage of ingestion, inside the phagocytes, was replaced by an avidity for alcoholic eosin under the influence of phagocytosis. Plato found that bacteria which failed to stain with neutral red outside phagocytes take this dyestuff when ingested. This latter reaction is not, however, a criterion of death but is due rather to the acid digestive vacuole that surrounds all ingested particles within a leucocyte.

All these studies although interesting from the standpoint of the mechanism of phagocytosis give little information as to differences which may exist between living and dead bacteria outside phagocytic cells. In fact, Bordet noted specifically that the destruction of bacteria by means of an immune serum did not lead to change in staining reaction as tested by the eosin-methylene-blue combination.

Congo red, in the experience of Henrici, stains dead but not living bacteria and Seiffert has found that gentian violet in the presence of serum or deutero-albumose does likewise. In this latter case the protein apparently prevents the entry of the stain into bacteria except when they are rendered more permeable by death. Methylene blue has also been used with indecisive results (Fulmer and Buchanan; Bickert) in differentiating living from dead bacteria. This dye has also been employed in combination with neutral red (Ruzicka).

Of all stains employed in differentiating living from dead bacteria the most reliable although perhaps the least well known is the one proposed by Proca in 1909 and subsequently advantageously modified by Kayser. This procedure in the hands of the latter investigator involves a somewhat prolonged staining with Loeffler's methylene blue, followed by rapid exposure to dilute Ziehl-Neelsen's carbol fuchsin.

RELIABILITY OF THE PROCA-KAYSER STAIN AS INDICATIVE OF DEATH OF BACTERIA, YEASTS AND SPORES

The Kayser modification of the Proca stain consists in separating the two elements combined in the original stain and applying first, for from three to five minutes, Loeffler's alkaline methylene blue to a specimen that has been fixed in the ordinary manner by drying and flaming, followed by the application to the drained and rapidly washed specimen of a dilute (1:10) solution of Ziehl-Neelsen's carbol fuchsin for five to ten seconds only, followed by rapid washing in water. The time relations of this process are extremely important, although certain substitutes in the way of dyes to be used and the order of their application may be made within certain limits, as will be later discussed. It should be specifically pointed out that living bacteria dried and fixed in this fashion are still viable and serve as controls to bacteria that have been killed by the various methods to be enumerated (Ficker).

We have studied a considerable range of both Gram-negative and Gram-positive bacteria, including *Streptococcus*, *Bacillus anthracis*, *Diplococcus pneumoniae*, *Coryn. diphtheriae*, *Staphylococcus*, the avian tubercle bacillus and yeast (*Saccharomyces cere-*

visiae) among the Gram-positive forms, and *Vibrio cholerae*, *Ebert. typhi*, *Esch. coli*, *Klebs. pneumoniae* and *Bacillus proteus* among the Gram-negative forms.

The nucleated red blood cells of the pigeon, chicken and incidentally some other nucleated cells, such as those of the alligator, have been employed to demonstrate certain apparent relationships to bacteria, as will be later related.

The effect of death on the staining reactions of these bacteria has been extensively and thoroughly studied and on the supposition that the method of killing might have some effect on the result produced, the cultures have been killed in a number of different ways. Thus, the killing of bacteria by heat, by formalin, by phenol, by HgCl_2 , by gentian violet and by saponin, has been carefully studied, each with several of the forms enumerated above. Destruction of bacteria by means of normal serum as exemplified in the destruction of *B. anthracis* by normal rabbit serum which is due to the thermostable beta-lysins of Pettersson, and also the specific destruction by immune serums of the cholera vibrio and of the typhoid bacillus have been investigated. In cholera the destruction by an immune serum is recognized to involve visible disintegration (Pfeiffer's phenomenon) whereas in the majority of other bacteria, the cell may remain apparently intact though dead. Another form of bacterial destruction is that produced by means of a specific bacteriophage and in this regard the staphylococcus, *Coryn. diphtheriae* and *Esch. coli* have been investigated. It is well recognized (Bronfenbrenner, Bayne-Jones and Sandholzer) that phage action involves a swelling and granular disintegration that is not unlike that present in Pfeiffer's phenomenon. Investigations in this laboratory (Stone and Hobby) indicate that destruction of the diphtheria bacillus takes place principally when the culture is in its coccoid stage.

Death produced by any of the methods that have been outlined results in a change of staining reaction in the Proca-Kayser method, that is to say, whereas living bacteria in controls stain blue they tend to take the red stain after death. This color shift varies in degree and clearness and various stages of change appear ranging from the original blue to purple to pink to red, depending

not only on the organism that is involved but on the surrounding medium in which it is suspended when subject to the lethal influence, and possibly on the rapidity of death. Some of these variable factors in the staining method will be specified, but it may here be categorically stated that there is a change produced in bacterial death which is invariably in the direction of a shift from blue to red, and which follows death by whichever of the methods employed. It is scarcely necessary to state that the death of the bacteria is in every instance authenticated by cultures which are sterile or which show a greatly decreased number of organisms.

Among the limitations of the method the following should be noted:

Early cultures of bacteria, for instance typhoid bacilli, grown for only five hours frequently show many pink organisms instead of uniformly blue forms.

Cultures of from forty-eight to seventy-two hours of certain organisms, as for example anthrax and proteus, show many pink forms.

Suspensions or cultures that have stood in the icebox for a considerable period of time, although many may be living, show many pink organisms.

We do not regard these exceptional pink forms as indicating any failure of accuracy in the method, but simply as indicating that the cultures concerned actually contain a number of dead organisms.

These remarks may be amplified by two examples which to us are particularly illustrative as to what these apparent exceptions mean. We have studied rapidly desiccated cultures of three organisms, the bacillus of anthrax, the pneumococcus and the typhoid bacillus. Previous studies have assured us that although such dried preparations are living when tested by subculture (Gay, Atkins, and Holden), the great majority of the bacteria, in fact from 91 to 93 per cent, have been destroyed, and we are not surprised to find that such preparations contain a majority of pink forms with an exceptional blue one. *Bacillus anthracis* from stock cultures, uncapsulated and without spores in the early

stages of its growth (four to six hours in saline suspension from agar) is invariably a blue-staining organism. When examined in agar cultures aged from twelve to eighteen hours, prespores are visible and unstained inside the rods which are stained purple or pink. These latter findings were interpreted to mean that the vegetative phase of the cell is dead as soon as the spore is formed, a conception which we believe is new but wholly consistent with the accepted explanation as to the mode of formation of anthrax spores from the chromatin granules present in the cytoplasm (Eurich and Hewlett; Bayne-Jones and Petrilli).

Saline suspensions from agar cultures give better results both with living and dead forms than do bacteria grown in broth cultures. Bacteria killed after suspension in distilled water in which they are originally blue in color may not show as definite a transformation to the red forms as when suspended in saline. The presence of serum in the suspension tends to prevent the transformation to red forms after death. The granules of the cholera vibrio in Pfeiffer's phenomenon are purplish rather than red, a partial transformation which may be due to the rapidity of death.

The staining of spores by the Proca-Kayser method presents a special and interesting problem in itself which yields results that differ from those obtained with vegetative bacterial cells. As Proca and Danila have already described, living anthrax spores (presumably extra-cellular) fail to take the stain, whereas dead spores stain blue. Our experience would modify their statement only in saying that living anthrax spores whether intracellular (prespores) or extracellular, fail to stain by the Proca-Kayser method, or at best show a faint bluish areola. This areola may be modified by a pinkish adherent rim of dead vegetative protoplasm. Blue-stained spores are rarely seen (dead?) under normal conditions. When, on the other hand, anthrax cultures are autoclaved, the spores stain a deep blue as contrasted with the red of dead vegetative forms.

In our experiments the spores of *B. subtilis* reacted somewhat differently from those of anthrax. In the first place, the spores are more ovoid in appearance than those of anthrax and in the presumably living condition, more spores are stained uniformly

blue. In autoclaved cultures the spores are stained blue although not so intensely as are those of *B. anthracis*.

In general, the spores of anaerobic bacteria, so far as can be judged from cultures of *Clos. tetani*, *Clos. sporogenes* and *Clos. histolyticum* are in agreement with the results obtained with the anthrax bacillus except as regards their intracellular position.

ON THE MECHANISM OF, AND VARIATIONS, IN THE PROCA-KAYSER STAINING REACTION

The study of staining methods has been of enormous significance not only in its relationship to bacteriological identification and taxonomic distinctions, but as bearing on the fundamental questions of bacterial structure and bacterial physiology. It is the Gram stain in particular which has served as an extensive theme of study for many investigators whose various conclusions we do not plan to mention here. Furthermore, the penetration of bacteria by stains bears an intimate relationship to the process of disinfection by dyes and to dye therapy. And, again, the question of vital staining both of bacteria and of animal cells is obviously dependent on the use of bacterial stains. The changes in protoplasm that typify a change from life to death have largely been based on a study of dye absorption, as for example in the experiments of Kite and of Lepeschkin.

We have considered the indications afforded by the Proca stain, and by other stains to be mentioned, as regards their mechanism and their significance in representing changes in bacterial protoplasm in death as contrasted with life. The first impression that one would gain from the original methylene blue-eosin contrast stain of Bordet is that the change in avidity for one or the other of the dyestuffs involved is due to a crude change in the reaction of the bacterial protoplasm, that is to say, whereas living bacteria have an acidic protoplasm that naturally unites with basic dyes, the reaction changes with death toward a basic reaction which makes them more eager for an acid dye. Although this might seem in agreement when eosin is used as a counter stain, it would not apply to the use of carbol fuchsin, which is a basic dye, in the Proca stain.

In general the pH of the suspension medium, so long as it is on the alkaline side of the iso-electric point of most proteins, is not a factor in this shift of staining reaction, although some degree of interference with the development of a typical red color occurs when bacteria are killed by heat in buffer solutions at lower pH levels (4.3; 5.3). Similarly, since both dyes in the stain mixture are basic, i.e., since the cation is the colored portion of the molecule in each case, we cannot look upon this differential staining as resulting from reversal of electric charge during the process of death.

The shift from blue to red in the Proca-Kayser staining cannot be fully explained until the chemical changes incident to death are more fully understood. Since the effect of an alteration in pH or of reversal of electric charge on the staining substrate does not seem to operate, we may regard the reaction as incident to the denaturation of nucleoproteins during death, whereby, because of alteration in the number or kind of reactive groupings of the denatured protein molecules, affinity for both the methylene blue cation and the fuchsin cation is reduced. Fuchsin can more readily replace methylene blue owing to its greater staining ability and the greater permeability of dead bacterial protoplasm.

As bearing on this question and also as to other stains that might be employed, table 1, showing possibilities which give as good or nearly as good results as the original alkaline methylene blue followed by Ziehl-Neelsen's carbol fuchsin, is offered. Many other dyestuffs have been tested both as primary and as secondary stains with negative results. Those dyes which are included in parentheses give partial but not clear cut results in a shift from the blue after death. Carbol fuchsin, aqueous basic fuchsin and safranin are all equally good as contrast stains after methylene blue. Apparently the only dye which is as good as alkaline methylene blue is saturated aqueous methylene blue. Toluidine blue and thionin, although they give fairly good results when used as a primary stain, are distinctly inferior to the two varieties of methylene blue.

The stains as applied in the Proca-Kayser method do not of course indicate that dead bacteria will not stain with methylene

blue. As a matter of fact, we find that dead bacteria stain *more faintly* with methylene blue than do living bacteria. This should not be confused with a somewhat generally accepted fact (Eisenberg, Nicolle, Seiffert) that dyes *penetrate* dead bacteria better than they do living ones; nor again with the fact that certain dyes are reduced by living as contrasted with dead cells.

And, on the other hand, carbol fuchsin will stain living bacteria as well as dead ones. Its selective property of replacing methylene blue in dead, as contrasted with living bacteria, is apparently due to the fact that the dead cells are less deeply stained by the primary stain than are living ones. In fact, carbol fuchsin will

TABLE 1
Possible modifications of Proca's stain

PRIMARY STAIN	SECONDARY STAIN
Loeffler's methylene blue	Carbol fuchsin
Loeffler's methylene blue	Aqueous basic fuchsin
Loeffler's methylene blue	Safranin
Loeffler's methylene blue	(Bismarck brown)
Loeffler's methylene blue	(Alcoholic eosin)
Saturated aqueous methylene blue (Toluidine blue)	Carbol fuchsin
(Thionin)	Carbol fuchsin
	Carbol fuchsin

replace methylene blue even in living bacteria if applied for from fifteen to thirty seconds instead of five to ten. This technical difference is one of the points involved in correct application of the method itself.

The order of staining cannot be reversed, that is to say, if carbol fuchsin is applied first to dead bacteria, followed by methylene blue, they stain pale blue instead of pink or red. In other words, the carbol fuchsin replaces the methylene blue in dead bacteria apparently because dead protoplasm has failed to retain the original dye as well as when alive and because the fuchsin is recognizedly a much more active and rapid dye stuff for bacteria.

NEUTRAL RED AS A DIFFERENTIAL STAIN FOR LIVING AND DEAD BACTERIA AND FOR ANIMAL CELLS

We have already referred to the work by Plato which showed that bacteria that have been ingested by phagocytes are stained red whereas they are unstained outside the cell. Metchnikoff, although he mentions this reaction as indicative of the destructive effect of the process of phagocytosis on living bacteria emphasized that it indicates simply the formation of an acid digestive vacuole about the organism. In other words, it does not mean that the ingested bacteria are dead and the stain is not in any degree analogous to the Proca stain. A dilution of neutral red in saline solution of 1:8000 is almost invariable in its reaction on bacteria, in accordance with whether they are inside or outside phagocytes. The cells remaining in the surrounding fluid whether they be living or dead usually fail to take any stain or at most show a faint brownish, yellow color. Nor does it make any difference whether such bacteria are treated with normal or immune serum. There is one exception, in that anthrax bacilli outside of cells stain a faint pink in about 5 per cent of their numbers which probably indicates that they are becoming acid, which may or may not mean that they are about to undergo degeneration. On the other hand, bacteria of various kinds that have been taken up by phagocytes, whether living or dead, although at first unstained, rapidly take on a distinct brownish-red coloration provided that the cells in which they are ingested are still alive, as is indicated by the fact that they do not take a diffuse pinkish coloration with the red but may show fine granules (polymorphonuclear cells) or segregation vacuoles (clasmatoctes). We have studied this process both in the animal body and in the test tube and with exudates obtained from rabbits and from guinea pigs, and with exudates containing large proportions of polymorphonuclear or of mononuclear cells, with the results that have already been briefly summarized.

It will be recalled that both Mesnil and Bordet used a compound staining method consisting of methylene blue followed by alcoholic eosin to indicate the destruction of bacteria within

phagocytes, assuming that the dead microörganisms alone stain with eosin. In our experience the Proca stain is distinctly better but with neither of these staining methods have we obtained uniformly demonstrative results of the intraphagocytic death of bacteria. Red organisms can, to be sure, be found after varying intervals among the bacteria that have been phagocyted but they are difficult of demonstration as bearing on the universally accepted purpose of phagocytosis. We do not mean these remarks to indicate any invalidity in the generally accepted hypothesis that phagocytes do actually destroy living bacteria but simply that an initial change in staining reaction as preceding disappearance of bacteria within phagocytes is hard to demonstrate.

The yeast cell offers an interesting intermediary organism between bacteria and animal cells as regards staining by neutral red. Yeast cells take up neutral red in small amounts in their vacuoles. When yeast cells have been killed or when they die as a result of ingesting too great a concentration of neutral red, they become diffusely stained. This must mean that dead yeast cells, in contrast to dead bacteria, become definitely acid in reaction.

The effect of neutral red on nucleated red blood-cells

The reaction of bacteria within phagocytes to neutral red suggests interesting analogies with the staining of the nucleus of nucleated red blood-cells by this same dyestuff. We have made certain observations with alligator cells and have particularly studied pigeon and chicken red cells. The reaction of nucleated red blood cells to neutral red raises acutely the question of the existence of a true "vital" stain, whether for bacteria or for animal cells, in contradistinction to a staining which simply represents a stage in a process of injury. Although the experiments of Churchman would indicate that certain Gram-negative bacteria are alive and motile, after staining with gentian violet, and similar results are claimed for animal cells in several articles reviewed by Möllendorff, and in the experiments of Churchman and Russell with paramecium, the question of injury must still remain a debatable one. For example, we find under conditions specified below that dilutions of neutral red give a perinuclear dot stain in

chicken or pigeon cells in dilutions as great as 1:2,000,000, and stain the whole nucleus uniformly when used in concentration of 1:4000 to 1:8000. Neutral red hemolyzes and agglutinates these same cells in dilutions of from 1:400 to 1:1000, depending on the specimen of dye and the particular cells in question.

Although stated thus categorically the nuclei of red blood cells are not always stained by neutral red as indicated for reasons that are by no means clear. Thus, when saline washed cells are suspended in mixtures of isotonic (0.85 per cent) saline and serum, or in broth (pH 7.4) they do not stain; when washed and suspended in saline they do stain. Since we know that unbuffered saline soon becomes acid on standing (\pm pH 6) it was natural to assume that staining depended on the acidity of the medium. But when phosphate-buffered isotonic saline solutions ranging in pH from 4.3 to 8.5 were employed, the nuclei stained in all although more markedly in the more acid range. It is apparent by staining fixed preparations that the cells in the acid range of these buffered isotonic solutions are injured without presence of neutral red, but the reaction alone does not account for staining by neutral red in saline buffered at pH 7.4. They apparently are still injured when neutral red sufficient to stain the nuclei is added. It seems probable that the living red blood cell, particularly when suspended in the most favorable menstrum (broth or serum) reduces a certain amount of neutral red that may penetrate the cell. When the cell is injured by acid the dye penetrates more readily. At all events, staining of the nucleus by neutral red would seem to be a reaction of injury rather than a true vital staining. Nucleated cells when killed and fixed with formalin do not stain with neutral red.

These and many other staining experiments were originally begun in an attempt to find some staining reaction which might be indicative of the sensitization process produced by immune serum but in this respect they have been completely negative.

We are interested here in comparing this, and other staining methods applied to nucleated red blood cells, either in their natural condition or after hemolysis by various methods, as compared with their effect on dead bacteria to which these red blood

cells are to some extent analogous. For this purpose red blood cells have been hemolyzed by a specific immune serum, or rather a specific immune serum heated to 56° and reactivated by guinea pig alexin, or else laked by the addition of ether, or water, or saponin. Cells destroyed by any one of these methods present with minor variations the same appearance, that is to say, they lose their hemoglobin and the cytoplasm disappears more or less completely. It is characteristic of all methods of hemolysis, however, that the nuclei remain relatively intact for longer periods of time although they tend to swell and become otherwise distorted. When lysed nucleated blood cells of the pigeon produced by the various methods enumerated are stained with neutral red the swollen nuclear material surrounded by fragments of cytoplasm fails to take the dye in the characteristic fashion. In other words, it has lost its power of natural staining with neutral red, being described in our protocols as "tinted" instead of reddish. In other words, the remnants of killed (laked) blood cells change in their reaction to neutral red whereas bacteria when killed by the methods we have enumerated do not; the latter are still unstained outside the cell and are stained by neutral red almost as well as are living bacteria within the phagocyte.

When hemolyzed nucleated pigeon cells are stained by either the Proca or the Wright stain, they show on the other hand a complete analogy to the change in staining affinity demonstrated by bacteria. Whereas the original intact cells of the pigeon show a blue nucleus with either Wright or Proca stain, and a coppery red cytoplasm with Wright stain and a yellowish protoplasm with Proca stain, when hemolyzed the nuclear remnants change from blue to pink as do bacteria.

Red blood cells are known not only to be phagocyted but to be destroyed in phagocytes. It is known that macrophages exercise a selective action in this regard as contrasted with polymorphonuclear cells which are concerned primarily in disposing of bacteria. It has perhaps been taken for granted that the destruction of red blood cells within phagocytes is essentially a process of hemolysis but a careful study of the matter by these staining methods in which we are interested indicates certain distinctive differences

from extracellular hemolysis which are of interest. When examined by the Proca or Wright stain, nucleated pigeon cells in macrophages of the rabbit or guinea pig gradually disintegrate through a shrinking of the cytoplasm and swelling of the nucleus which latter part of the cell becomes a brownish pink in color in contradistinction to the normal blue. When examined by the neutral red stain it is seen that the cell cytoplasm, which is somewhat brownish yellow in color within the phagocyte, slowly shrinks. The nucleus, at first unstained, takes the neutral red as it does in saline suspension and gradually swells but continues reddish in color as contrasted with what happens to it outside the cell where it finally fails to stain.

SUMMARY AND CONCLUSIONS

The Proca-Kayser differential stain, applied to living as contrasted with dead bacteria, has been extensively studied and its accuracy confirmed. Provided certain specified conditions as to order and duration of application of each of the stains involved (methylene blue then carbol fuchsin) are observed, the stain clearly indicates whether the bacteria were alive (blue stained) or dead (purple to red) when placed on the slide on which they are subsequently dried and fixed by heat, in the usual fashion. The apparent exceptions, in failure of a shift in staining on the part of individual organisms, are found to be simply confirmatory of the accuracy of the method.

Death as produced by physical or chemical agents, by bacteriophage, or by serum results in each instance in a similar shift in staining.

Gram-positive and Gram-negative bacteria, acid-fast bacteria and yeast all show a similar shift in staining reaction. The distorted but relatively resistant nucleus of nucleated red blood cells hemolyzed in different ways shows a corresponding shift from blue to red when stained by the Proca method.

Living bacterial spores, whether intracellular or extracellular, take the faintest if any stain by the Proca method; when dead they stain blue.

Certain substitutions in either the primary or the secondary dye

stuff employed in the Proca-Kayser method are possible as explained in the text.

We have met with little success with the methylene blue-eosin contrast stain employed by Mesnil and by Bordet to show similar changes in bacteria within phagocytes. The Proca stain, although better, fails to show convincingly that phagocytes actually change live to dead bacterial protoplasm.

The precise nature of the change that bacterial protoplasm undergoes in death still eludes us. It is not simply a crude change in reaction of the bacterial protoplasm or reversal of electric charge in the staining substrate. Apparently changes in permeability of the protoplasm and in the reactive groupings of the protein are involved.

Neutral red fails for the most part to stain bacteria until they become ingested by phagocytes. The intracellular stain is not however indicative of bacterial death but rather of the acidity of the digestive vacuoles. The nuclei of chicken or pigeon red blood corpuscles also take neutral red in great dilution. For reasons that are given this staining reaction is interpreted as indicative of injury to the cell rather than as a true vital staining.

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THE FAILURE OF BACTERIUM COLI FROM HUMAN FECES TO GROW AT 46° IN THE EIJKMAN OR THE BULÍR TESTS

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INTRODUCTION

The Eijkman test (1904) is designed to detect fecal pollution of water. Water is inoculated into glucose-peptone broth which is incubated at 46°, and gas production is assumed to indicate fecal pollution from warm blooded animals. *Bacterium coli* and *Bacterium aerogenes* strains of non-fecal origin or from feces of cold-blooded animals are not supposed to grow, or at least not to produce gas under these conditions. Although much has been written in its favor and much against the test, it would seem that the fundamental question as to whether or not most of the colon bacilli from human feces are able to grow and produce gas at a temperature of 46° has not been satisfactorily answered. A review of both sides of the question will be found in the papers of Leiter (1929), of de Magalhães (1932), and of the authors (1930). The latter, by using the dilution method, showed that in many samples of feces, at least half of the bacteria capable of producing gas from lactose at 37.5° refused to produce any at all from glucose at 46°, and that in some samples of feces very few, even less than 1 per cent of the fecal *B. coli*, were able to produce gas under the conditions of the Eijkman technique.

The Bulír test (1907) is a modification of the Eijkman test, and consists in using a mannitol-peptone beef infusion broth at 46°. According to Minkewitsch and Trofimuk (1928), Minkewitsch, Trofimuk and Wedenjapin (1928), and Minkewitsch (1929, 1930a; 1930b; 1932) those fecal strains, which are heat labile in glucose broth, will produce gas from mannitol broth at 46°, thus making

the Bulfr test better than the original Eijkman method. Jungelblut (1921) was also favorable to the Bulfr test.

In going over the published work on these tests one cannot but be struck with the contradictory results. Some observers have found that all strains isolated from feces grow and produce gas at 46°. Others have found that only a small percentage will grow. Still others have found that they grow in mannitol but not in glucose broth. Such results can hardly be explained by chance. Technique must play a part.

In some cases small amounts of liquid were used, and in others hundreds of cubic centimeters; in some cases hot-air incubators and in others water baths were used. Now it is obvious that if large amounts of media are employed, all parts of the culture flask, when inoculated at room temperature or less, do not reach 46° for some time. Certainly some portions of the flask will remain lower than 46° for a longer time than others. In other words, one may be inoculating bacteria into media at about 20°, which temperature is increased irregularly in different parts of the flask until 46° is reached. Controlled temperatures are impossible by such methods. Smaller tubes will of course reach a uniform 46° temperature much sooner, but actually to reach 46° in all parts of the tube in the minimum length of time, a water bath incubator and small tubes are much to be preferred to flasks and hot-air incubators.

It occurred to the authors that the divergent results may possibly be explained by the above considerations, inasmuch as the bacteria which would be killed at 46° might possibly, in the course of a few generations, accustom themselves to a few degrees higher temperature than their previous thermal inhibition points. It was to investigate this point, to consider the possible superiority of the Bulfr over the Eijkman method, and to confirm or reject our former conclusions that the Eijkman test fails to detect a large part of the fecal *B. coli*, that the following work was done. This study was completed in the summer of 1932.

A short time ago another modification of the Eijkman test was proposed (Williams, Weaver and Scherago, 1933a) which is said to overcome some of the objections to the original method.

Since the data were not published in full until March, 1933 (Williams, Weaver and Scherago, 1933b) we have not yet attempted to test this method. We strongly urge, however, that, if the method be used to indicate fecal pollution, a large series of determinations on many samples of feces be made. It is necessary not only to find that some of the *B. coli* from feces grow in any test, but that all or nearly all of them do,—at least as many as grow in standard lactose broth. The use of separate, isolated strains, in our opinion, gives far less information than do counts by the dilution method. Not only does one obtain no quantitative data by the use of separate isolated strains, but it is very probable that a single cell may refuse to multiply at 46°, whereas the progeny of this one cell in a culture having cells of different physiological ages (Sherman and Albus, 1923) may contain one or more cells able to develop, and a heat tolerance be built up by the culture.

EXPERIMENTAL

The media used were as described by the authors of the tests in question. They were prepared in such concentration that after the water was added, the final concentrations of nutrients were as follows:

Eijkman—glucose 1.25 per cent, Difco peptone 1.25 per cent, NaCl 0.625 per cent;

Bulir—mannitol 0.66 per cent, Witte peptone 0.55 per cent, NaCl 0.33 per cent; to beef infusion inoculated with *Bacterium aerogenes* twelve hours to destroy muscle sugar, brought to pH 7.0, and neutral red added.

Lactose broth—lactose 1.0 per cent, Difco peptone 1.0 per cent, Leibig's beef extract 0.3 per cent, NaCl 0.5 per cent, adjusted to pH 7.0.

These media were inoculated, as described in our previous work (1930), in Durham fermentation tubes from the same dilutions at the same time and with the same pipettes. A replicate set of five for each decimal dilution of both glucose and mannitol media was placed at once in a water bath at 46° connected with a DeKhotinsky thermoregulator and an efficient stirring pump.

Two other sets were placed in a water bath filled with fresh tap water, also with a stirring pump and thermoregulator, and brought slowly to 46° (five hours), after which the temperature was maintained at 46° by the same methods as above. In no case was there ever visible a trace of gas in five hours in any of the tubes of glucose or mannitol media. The thermometers were checked with Bureau of Standards thermometers. The fifth set (lactose broth) was incubated at 37.5° in a hot-air incubator. At the end of twenty-four and forty-eight hours, the tubes showing gas were recorded and the most probable number of *B. coli* per gram of feces was determined by each method, using the forty-eight hour figures and the tables of Halvorson and Ziegler (1933a; 1933b). The number of millions of *B. coli* per gram is shown in table 1 in order of determination. Almost every sample represents a separate individual, there being only five on which the determination was repeated, when new samples of feces could not be obtained. The results of the whole sample in which all the tubes in any one set were all negative or all positive were discarded. A new sample was obtained, and run again using higher or lower dilutions.

The mean in table 1 for each of the 5 methods shows the most probable number of *B. coli* per gram of a combined mixture of equal quantities of all the samples of feces. Clearly the lactose broth at 37.5° shows more *B. coli* than do any of the other 4 methods. That all the positive tubes in the lactose broth series actually contained *B. coli* may be taken for granted from considerations given in a previous publication (1930), since confirmed by more accurate methods (Skinner and Brudnoy, 1932).

The different methods of determining *B. coli* were compared as follows: Figure 1 shows the value of lactose broth in relation to glucose broth at 46°. To arrive at these values, the ratios of the number of *B. coli* were calculated as determined in lactose broth at 37.5° to the number in glucose broth at 46° for each sample of feces. These are shown in figure 1, the heavy line above the ± 1 mark. Each point will show, for a sample of feces, the number of *B. coli* by the lactose-broth method in a given volume, when the number in the same volume according to the Eijkman test is 1.

Below the ± 1 line are shown samples values for those samples of feces which gave a higher number per cubic centimeter by the Eijkman test than by the lactose broth test at 37.5°. These

TABLE 1

Number of Bacterium coli in separate samples of human feces determined by dilution technique by means of five different media

(Millions per gram)

-
- A. Glucose broth at 46° (Eijkman method): 17.1, 1.71, 54.2, 0.231, 0.020, 0.107, 0.045, 0.45, 0.169, 162, 943, 23.1, 2.31, 3 29, 20, 0.78, 0.20, 79.2, 1.30, 7.92, 4930, 0.0231, 13, 1.07, 79.2, 109, 2400, 0.20, 2.31, 1300, 792, 0.67, 24, 700, 0.78, 4.93, 792, 329, 21.7, 79.2, 10.6, 0.41, 49.3, 21, 13.9, 0.78, 329, 3490, 0.2, 1.69, 21.7, 542, 1300, 171, 493. Mean 351.651
- B. Glucose broth at room temperature brought to 46° in five hours, thereafter at 46° (modified Eijkman method): 10.9, 1.2, 162, 34.9, 0.231, 0.493, 2.21, 54.2, 2.21, 162, 1710, 45, 34 9, 17.1, 231, 1.69, 4 25, 542, 27.8, 13, 2310, 54.2, 13, 23.1, 79.2, 27.8, 5420, 1620, 32.9, 3290, 5420, 2 4, 141, 1410, 7, 542, 493, 329, 456, 240, 349, 0.78, 171, 493, 109, 0.78, 9180, 9180, 0.20, 4.93, 49.3, 1620, 9180, 49.3, 1300. Mean 1030.073
- C. Mannitol broth at 46° (Bulfr method): 0.078, 2.31, 0.078, 0.78, 0.107, 0.7, 0.329, 0.7, 3.29, 34.9, 1710, 84.0, 10.9, 24.0, 493, 0.78, 2.31, 345.0, 45.6, 22 1, 2310.0, 7.92, 109, 49.3, 32.9, 79.2, 792, 0.2, 3.29, 700, 2780, 1.71, 32.9, 792, 4.56, 3.29, 1300, 493, 49.3, 141, 141, 0.45, 130, 109, 171, 0.68, 1090, 3490, 0.41, 4.93, 49.3, 1620, 792, 32.9, 493. Mean 374.313.
- D. Mannitol broth at room temperature, brought to 46° in five hours, thereafter at 46°, (modified Bulfr method): 22.1, 1.3, 5.75, 4.93, 0.792, 4.93, 2.40, 7.92, 54.2, 54.2, 792, 231, 7, 349, 3490, 2.31, 2.31, 918, 79.2, 10.9, 4930, 91.8, 23.1, 130, 240, 13, 3490, 349, 79.2, 3140, 16200, 3.49, 49.3, 1300, 24.0, 79.2, 1710, 1300, 1300, 349, 70, 0.20, 171, 171, 141, 3.29, 5420, 5420, 0.20, 4.93, 49.3, 1620, 5420, 32.9, 231. Mean 1083.566
- E. Lactose broth at 37.5°: 13, 2.21, 54.2, 34.9, 17.1, 3.29, 34.9, 17.1, 130, 171, 493, 493, 49.3, 24, 16,200, 1.28, 23.1, 918, 109, 17.1, 7920, 91.8, 70, 171, 141, 171, 5420, 1620, 349, 3290, 16200, 5.42, 49.3, 3490, 91.8, 109, 32.9, 1300, 456, 171, 349, 0.2, 918, 700, 109, 2.31, 9180, 16200, 4.56, 10.9, 79.2, 1620, 3490, 171, 329. Mean 1693.052
-

were calculated by dividing the number per cubic centimeter according to the Eijkman test by the number per cubic centimeter when the lactose broth at 37.5° was used. The heavy line, showing the comparison of the Eijkman and lactose broth meth-

ods includes the data from our former paper (1930) recalculated from the tables of Halvorson and Ziegler (1933a; 1933b), with all values omitted, in the interest of greater accuracy, which were obtained from 5-5-5 or 0-0-0 results. The light line is likewise a comparison of the results with lactose broth at 37.5° and glucose broth brought to that temperature gradually in five hours.

If it were true, that the Eijkman test or the test as we have modified it, allowed as many of the fecal *B. coli* to produce gas

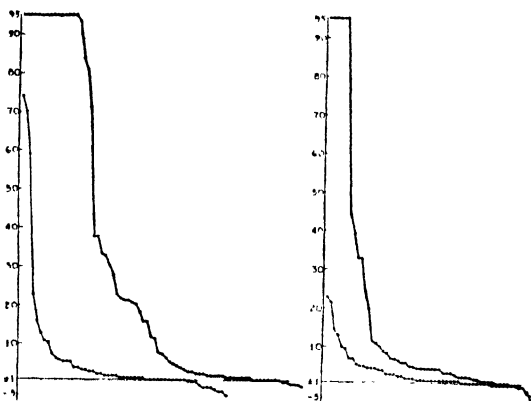


FIG. 1

FIG. 2

FIG. 1. COMPARISON OF THE EIJKMAN TEST WITH LACTOSE BROTH METHODS (HEAVY LINE), AND OF THE EIJKMAN TEST MODIFIED SO AS TO ALLOW FIVE HOURS AFTER INOCULATION FOR THE 46° TEMPERATURE TO BE REACHED WITH LACTOSE BROTH METHODS (LIGHT LINE) FOR THE DETERMINATION OF *B. COLI* IN FECES

FIG. 2. COMPARISON OF THE BULIR TEST WITH LACTOSE BROTH METHODS (HEAVY LINE) AND OF THE BULIR TEST MODIFIED SO AS TO ALLOW FIVE HOURS AFTER INOCULATION FOR THE 46° TEMPERATURE TO BE REACHED WITH LACTOSE BROTH METHODS (LIGHT LINE) FOR THE DETERMINATION OF *B. COLI* IN FECES

as does lactose broth at 37.5°, we should expect for each curve approximately the same number of points above the ± 1 line as below it. The areas above and below should also be equal. The curves should be horizontal, symmetrical, and S-shaped, centered at the middle of the abscissa, on the ± 1 line. That these curves do not in any way agree with this may be seen at once. They show definitely that neither method of performing

the Eijkman test can be depended upon to detect anything like all the *B. coli* of fecal origin.

Figure 2 for the Bulir test likewise shows that mannitol broth at 46° can be expected to miss some of the fecal *B. coli*, particularly if the 46° is reached immediately after inoculation. The heavy line represents the number of *B. coli* determined by lactose broth at 37.5° divided by the number according to the Bulir test at 46° from the start. The light line represents the quotient of the

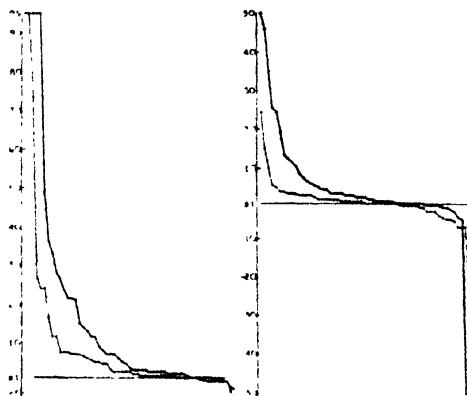


FIG. 3

FIG. 4

FIG. 3. COMPARISON OF THE EIJKMAN TEST WITH THE TEST MODIFIED TO ALLOW FIVE HOURS TO BRING THE INOCULATED MEDIA TO THE 46° TEMPERATURE (HEAVY LINE), AND A COMPARISON OF THE BULIR TEST WITH THE TEST LIKEWISE MODIFIED (LIGHT LINE) FOR THE DETERMINATION OF THE NUMBER OF *B. COLI* IN FECES

FIG. 4. COMPARISON OF THE EIJKMAN TEST WITH THE BULIR TEST (HEAVY LINE) AND WITH THE SAME TESTS MODIFIED TO ALLOW FIVE HOURS TO BRING THE INOCULATED MEDIA TO THE 46° TEMPERATURE (LIGHT LINE) FOR THE DETERMINATION OF *B. COLI* IN FECES

number determined by lactose broth at 37.5° divided by the number determined in mannitol broth inoculated at room temperature and brought to 46° in five hours.

Figure 3 shows the advantage of allowing time for the 46° to be reached. The heavy line represents the ratio of the numbers of *B. coli* at 46° in glucose broth, five hours being taken to reach this temperature, to the numbers in glucose broth held at 46° from

the start. The light line represents the same thing for the mannitol broth. It is evident that many cells which would fail to produce gas at 46° can do so if brought gradually to that temperature in culture media. This is particularly true in the Eijkman test. Possibly we have here a partial explanation for the divergent results of investigators as to the worth of the Eijkman test.

The heavy line of figure 4 was plotted by dividing the number of *B. coli* determined in mannitol broth at 46° by the number determined in glucose broth at 46°, that is, the ratio of Bulfr numbers to Eijkman numbers. The light line represents the ratio of the modified Bulfr to the modified Eijkman. While it can be seen that the Bulfr test as well as its modification may possibly allow some *B. coli* to produce gas which would not do so in the Eijkman test, the results do not indicate a very outstanding or consistent superiority. A comparison of the curves in figures 1 and 2 also shows the same thing. Next to lactose broth at 37°, the modified Bulfr test seems to be the best for gas production by fecal *B. coli*.

Nearly all investigators agree that the *B. coli* isolated from cold-blooded animals are generally unable to produce gas at 46°. Leiter (1929) and Magalhães (1932) in particular have found this to be true. Minkewitsch and Trofimuk (1928) have presented data which they interpret as showing the same thing for the Bulfr test. Magalhães isolated 65 strains from 7 species of fish, 2 of reptiles and 3 of batrachians; only 2 strains, isolated from fish (*Barbus bocagei* Steind.) produced gas under the conditions of the Eijkman test. We have utilized the dilution method with the feces of a few fish, and were not able to confirm the results of the authors cited above. Since our work was limited in amount, we do not as yet wish to do more than suggest that the possibility remains that the Eijkman test may not under all conditions eliminate the *B. coli* whose origin is cold-blooded animals any more than it does the strains from human feces. Further work utilizing the quantitative dilution method is contemplated.

CONCLUSIONS

These results offer additional evidence that the Eijkman test, if actually carried out at 46°, will fail to detect many of the *B. coli*

from human feces. The same is true for the Bulir test. They show further that some individuals harbor many more thermolabile strains than others. About one-third of the samples show a normal curve when the number determined by the Eijkman (or Bulir) methods is compared with that of the standard lactose broth methods, as seen in the heavy lines in figures 1 and 2. The other two-thirds, however, show a decided bias in favor of the lactose broth.

If the inoculated glucose or mannitol-broth tubes in the tests are increased slowly in temperature, simulating the conditions which obtain in portions of large flasks in hot air incubators, the bias in favor of lactose broth is considerably less, especially in the case of the mannitol broth. Even so it is evident that neither method of using the test will detect all the *B. coli* strains. Slightly less than one-half of the samples are in favor of lactose broth at 37.5°, beyond the normal experimental variation to be expected if lactose at 37.5° were as good as glucose (or mannitol) at 46° brought to that temperature in five hours. This can be seen in examining the light lines in figures 1 and 2.

The results as indicated in figure 3 show further that the Eijkman and Bulir tests, if actually carried out at 46° from the start, allow fewer *B. coli* to produce gas than if brought to that temperature gradually, after inoculation, which is, in effect, what many experimenters have done. This, in part, explains the conflicting conclusions of many authors as to the worth of the tests. However, even if the temperature is gradually adjusted to 46°, many of the human fecal *B. coli* are not detected by the Eijkman or Bulir tests.

Some advantages of the Bulir over the Eijkman tests were noted, less at 46° than at room temperature brought to 46° in five hours. This is shown in figure 4.

SUMMARY

1. By means of the dilution method, the values of 5 different techniques for determining *B. coli* were compared.

2. The Eijkman and the Bulir tests failed to detect nearly as many *B. coli* as did the lactose broth at 37.5° method. To a less

extent this was true when the 46° of the Eijkman and Bulír methods was reached in five hours by a gradual increase in temperature.

3. A partial explanation for the conflicting results in the literature on the Eijkman test is proposed. It is possible that the large amounts of liquid in hot-air incubators, used by many investigators, allowed, in the course of a few generations, some, of the *B. coli* to adapt themselves to the 46° of the Eijkman and Bulír tests. Data showing this are given.

4. The Bulír test has some advantages over the Eijkman test, but neither can be relied upon to detect fecal pollution.

5. The fact that forty-eight hours incubation is necessary for the Eijkman or the Bulír test was confirmed.

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INTERCHANGE OF BACTERIA BETWEEN THE FRESH WATER AND THE SEA

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Burke and Baird (1931) have presented experimental evidence that many fresh water and land bacteria can exist and multiply in an environment having the salt concentration of the sea. If fresh water bacteria survive in the sea, it follows that at least some of the bacteria in the sea should survive in fresh water, and that there should occur, therefore, an interchange of bacteria between the sea and the fresh water and land environment. The experiments described in this paper were designed to produce further evidence as to the possible exchange of bacteria between the land and the sea.

This work was done in the summer of 1931, at the Hopkins Marine Station, Pacific Grove, California. At this time of the year the streams entering the Monterey Bay and nearby ocean have ceased flowing or are greatly reduced, so that the shore waters cannot be considered brackish.

EXPERIMENTAL

The medium used, adjusted to pH 8.0, consisted of 5 grams peptone, 3 grams beef extract, 30 grams gelatin, 100 cc. potato extract, 15 grams agar, and 900 cc. sea water or tap water. Material from various fresh water, soil, and marine sources was plated out on the two media and counts made on successive days.

The marine material consisted of sea water free of plankton, taken near shore, sea water taken a mile or more from shore rich in plankton, the same water incubated for two weeks, stomach contents of free-swimming and bottom fishes, sea water near the mouth of a small stream, decayed fish, and water from about a

decayed fish. In comparing growth on the two kinds of media, it was found that numbers varied from one to a thousand times as many and that colonies appeared sooner and were usually larger on the homologous medium. With the exception of the intestinal contents of fishes, the marine material never gave counts on the homologous medium of more than 15 times the count on the heterologous medium.

The fresh water and soil material consisted of water from two streams above high tide, decayed marine fish placed in streams for two to six weeks, the same fish left in the laboratory two weeks, water surrounding the same fish, and soil in front of laboratory. With some material the plate counts were the same on the two kinds of media, with others up to 40 times as many colonies appeared on the homologous medium. The colonies appeared earlier on the homologous medium and there was noted a difference in size, pigment production, and type of colony formation.

Anaerobic shake-agar cultures made from material heated to destroy vegetative cells gave comparable results. In general more colonies grew on the homologous medium and the anaerobic line was nearer the surface.

In all the sea and fresh-water material examined there were bacteria that grew in the heterologous medium. Whether the counts on the two kinds of media were identical or varied greatly depended on the source and nature of the material. In some specimens, the predominating organism grew equally well on the two kinds of media, and the counts were practically identical.

The experiments so far reported demonstrate that both fresh water and the sea contain many bacteria that will grow in the heterologous environment. They do not indicate whether certain species fail to grow. Cells of all the species present might grow and the counts differ. Also the counts might be identical and different species be present, as pointed out by Lipman (1926).

A side-by-side comparison of plates of fresh water and sea water on the two kinds of media suggested that different organisms were appearing on the heterologous agar as well as that some were not growing. If this were true, then the effect of the

changed environment would be greater than indicated by the comparative counts. We therefore decided to determine the effect of salt on pigment production and the effect of the heterologous environment on the viability of the cells of pure cultures. Burke and Baird showed that cells of a species vary in their tolerance for salt. It occurred to us that the reduced counts on the heterologous agar might be caused by the more sensitive cells of each species not growing, rather than by certain species failing to grow.

Staph. aureus, *Sarcina lutea*, *Pseudomonas pyocyanca*, *Pseudomonas fluorescens* and *Serratia marcescens* were grown in 1 to 6 per cent salt. Salt had no effect on the pigment production of *Staph. aureus* and *Sarcina lutea*. Pigment production by *Serratia marcescens* was retarded by 2 per cent salt and never reached its maximum in 3 per cent salt. Pigment production by both species of *Pseudomonas* was slight in 1 and 2 per cent salt and inhibited in 3 per cent salt. The results were the same on extract agar and potato extract agar. Smith (1933) has shown that salt affects the pigment production, morphology, and colony formation of *Bacillus megatherium*. In plating out soil on sea-water agar we noted that salt checked the "spreader" type of colony.

It is evident from these results that it is not possible by a comparison of colonies on the two kinds of media to determine whether some species have failed to grow on the heterologous agar.

To determine whether the reduced counts on the heterologous agar could be due in part at least to the failure of the most sensitive cells of each species to grow, twenty-four-hour cultures of seventeen species of bacteria isolated from sea water were plated out on the two kinds of agar. All the species grew on the salt-free agar but all the cells did not. With some species the counts on the heterologous medium were reduced slightly, with others more than 50 per cent. We found no species, either fresh water or marine, that failed to grow on the heterologous medium. The reduced counts on the heterologous medium of the material we examined were due, in part at least, to the failure of the more sensitive cells to grow. What percentage of bacteria will fail to grow on the heterologous agar remains to be determined.

Since salt affects spore formation, it occurred to us that the fate of spore-bearing organisms in a changed environment such as occurs between land and sea might depend upon the effect of salt on the formation and germination of the spores (Curran, 1931; Smith, 1933).

A series of experiments with *B. subtilis* demonstrated that a salt concentration equal to that of the sea causes an increase in the ratio of spores to vegetative cells. This occurred in agar, broth and water. Further experiments on spores freed of vegetative cells by heat demonstrated that spores of this organism will develop in a salt concentration greater than that of the sea. The fate of some spore-bearing organisms in the sea apparently does not depend upon the effect of the salt on the formation and germination of the spores.

The experiments described suggest that fresh water bacteria may be able to maintain themselves in the sea. There is considerable evidence that they can maintain themselves in brackish water. To determine whether fresh water forms could be isolated from the sea, bacteria were isolated from decayed fish and water in a small stream flowing into the ocean and from decayed fish and sea water 50 feet to one side of the mouth of the stream. One hundred and five cultures were isolated over a period of several weeks and studied as regards staining, morphology and growth characteristics. After duplicates were eliminated there were left fourteen species from the sea and eight from the stream. When these were compared, five species appeared to be common to the stream and to the ocean near the mouth of the stream. The dilution of the sea water by the stream was negligible.

DISCUSSION

Since many fresh-water bacteria can survive and carry on their activities in salt concentrations equivalent to or greater than that occurring in the sea, we should expect to find that the bacterial flora along the sea shore is enriched by additions from the land and fresh water entering the ocean. If such forms survive in the ocean, they will undoubtedly survive when carried back to the fresh water environment. Our experiments favor

the view that such an interchange of bacterial species between the sea and fresh water occurs. How extensive this interchange is, and whether it is greater than with higher forms of life, remains to be determined. Workers in marine bacteriology can, by keeping in mind the possible interchange of bacterial forms between land and sea, avoid some of the mistakes made by workers in other groups.

CONCLUSIONS

Bacterial counts of fresh water and sea water are usually but not always reduced when the material is plated out on a heterologous medium as regards salt. The reduction in counts is due in a large part at least to the fact that the more sensitive cells, of many if not all species, fail to grow. The more resistant cells of many species grow in the heterologous environment.

A salt concentration equal to that of the sea causes an increase in the rate of spore formation of *B. subtilis*. These spores will develop in the same salt concentration.

Changes in salt concentration affect the characteristics of some species. Suspected new species of fresh water and marine bacteria should be studied in both a fresh-water and marine environment. To compare the characteristics of marine bacteria on sea-water agar with the characteristics of fresh water bacteria on fresh water agar is inadequate for the description of new species.

The writer wishes to acknowledge his indebtedness to Dr. W. K. Fisher and Dr. Van Neal for placing the facilities of the Hopkins Marine Station laboratory at his disposal.

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AN UNKNOWN FACTOR STIMULATING THE FORMATION OF BUTYL ALCOHOL BY CERTAIN BUTYRIC ACID BACTERIA¹

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The question of growth stimulants for microorganisms has long been one of the most interesting and widely discussed subjects of biological research. The widespread occurrence of growth-stimulating factors has been well demonstrated, and a wide variety of microorganisms have been shown to be affected by such factors. Peskett (1933) has recently given a general review of the subject but certain aspects dealing with the occurrence of stimulants in plant materials should be emphasized. Growth-factors have been found in lemon juice, carrots, potatoes, spinach, radishes and many other plant tissues. The organisms affected by some of these factors include yeasts (Robertson and Davis (1923)) and bacteria. Among the latter are butyric acid bacteria (Ruschmann and Harder (1931)), hemolytic organisms (Morgan and Avery (1923)), staphylococci (Leichtentritt and Zielaskowski (1922)), streptococci (Thompson (1929)), the pneumococci (Thjötta and Avery (1921), Kollath (1926), Kopp, (1927)), and the tubercle organisms (Uyei (1930)). Little is known about the rôle played by the stimulating factors or about their actual chemical nature. Thompson (1929) suggests that the growth-promoting action of potato extract is due to its nitrogenous food constituents rather than to an "accessory growth factor." Uyei (1930) on the other hand, investigating the stimulating action of potato on the tubercle bacillus, found that a protein preparation of potato

¹ This work was supported in part by a grant from the special research fund of the University of Wisconsin.

had no growth-promoting activity. He also found that the known carbohydrate constituents of the potato had slight stimulating effects which could not, however, be compared with that brought about by the extract itself.

In a previous paper (McCoy, Fred, Peterson and Hastings (1930)), it was reported that many butyric acid bacteria grew well and formed solvents (butyl, alcohol, etc.) on potato mash, but did not give such results on corn mash and other substrates. The present paper deals with the unknown factor which is responsible for this difference in fermentation and with the general distribution in nature of this factor.

EXPERIMENTAL

Cultures and media. Pure cultures of the anaerobic butyric-acid-forming bacteria were used, and according to bacteriological tests were free of contaminating forms. The detailed description of these organisms has not been completed and for the present the bacteria are listed by number. While all of these cultures are butyric-acid-producing forms, they have not been typed within the group and therefore some of them may be duplicates. Cultures 19, 21, 22 and 25 as listed in a former paper (McCoy, Fred, Peterson and Hastings (1930)) and in addition, cultures 36, 37, 38 and 39 were employed.

The media used were as a rule mashes containing 4 per cent of the grain or tuber calculated on the dry basis. Speakman's (1923) peptone-inorganic salt medium was used in a few experiments with 4 per cent glucose or purified starch as carbon sources. Throughout the early work 200 cc. of the medium in 250-cc. Erlenmeyer flasks were used. Later 8-inch test tubes (1 inch diameter) containing 35 cc. of medium were employed. Inoculation was made from a twenty-four hour culture of the organisms in corn mash. One per cent of inoculum was used in every case, and the cultures were incubated at 37°C.

The degree of stimulation was evidenced by turbidity of the supernatant liquid, by rapidity of gas evolution, by "head" formation, and by the characteristic butyl-alcohol odor. Throughout the investigation the conclusions arrived at by means of these

observations were checked by chemical analysis for butyl alcohol, ethyl alcohol and acetone.

Analytical methods. Ethyl and butyl alcohols were determined by Johnson's (1932) micro-method. Acetone was determined iodometrically.

Products of butyric acid organisms from corn and from potato. The products from these two materials are given in table 1. Corn is seen to be a poor substrate, very little (10 per cent) of the starch being fermented. The products were mainly acetic and butyric

TABLE 1
Products from fermentation of corn and potato
(Calculated for 100 grams dry matter)

CULTURE	MEDIUM	BUTYL ALCOHOL	ETHYL ALCOHOL	ACETONE ALCOHOL	TOTAL SOLVENTS
		<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>
21	Corn	1 01	0 09	1 04	2 14
21	Potato	13 60	0 49	0 97	15 06
36	Corn	0 18	0 89	0 82	1 89
36	Potato	12 97	0 62	1 16	14 75
22	Corn	0 46	1 06	1 28	2 70
22	Potato	12 68	1 32	0 92	14 92
25	Corn	0 23	1 01	0 97	2 21
25	Potato	1 55	0 91	1 16	3 52
19	Corn	0 19	0 87	0 65	1 71
19	Potato	1 21	0 98	1 08	3 27
38	Potato	2 90	1 12	1 16	5 15
37	Potato	2 81	1 27	1 01	5 09
39	Potato	1 41	1 15	1 04	3 60

acids and acetone, with very small yields of alcohols. All the organisms used showed a decided increase in butyl alcohol production from potato. This increase was correlated with an increased fermentation of starch, amounting in certain cases (cultures 21, 22 and 36) to 80 per cent. It is noteworthy that in all cases most of the increase in solvents was accounted for by butyl alcohol. The production of this substance was therefore taken as the criterion of stimulation in further work. Besides the five cultures tested on both corn and potato, three other cultures were tested on potato alone. None of these produced appreciable amounts

of butyl alcohol. The same lot of potatoes was used in all these fermentations so that the difference in butyl alcohol production can be explained only on the basis of strain differences. Although three of the cultures showed about the same response to potato, culture 21 was used for most of the later experiments because somewhat more data were available for it.

During the course of the investigation several varieties of white potatoes were fermented with culture 21. Three of the varieties were fermented vigorously, while two gave no better fermentations than corn mash. All samples of Wisconsin potatoes that have been tried brought about vigorous fermentations. These potatoes have, therefore, been used in the preparation of potato extracts for further work.

Fermentation of other starchy materials. Several other starch-containing natural products were fermented by culture 21 to determine whether the white potato was the only source of the stimulant. Only one of these materials, the thick-skinned sweet potato or yam showed any stimulative effect. It is curious that Jersey sweet potatoes, which are so similar to the yam showed no effect whatever. Wheat germ gave slightly higher yields of butyl alcohol than corn, but rice, oats and barley did not.

Effect of various supplements on the fermentation of corn by culture 21. It was thought that perhaps the failure of culture 21 to bring about a good fermentation of corn mash might be due to its inability to utilize the protein and starch of corn. Various modifications of the corn medium were therefore made in an attempt to improve the solvent production. The results are given in table 2. The addition of peptone (1 per cent) increased the butyl alcohol production to some extent, but higher concentrations added (3 per cent) had no further affect. An increased sugar concentration also increased the solvent production in corn mash, as is shown by the higher yield from corn which had been digested with malt diastase. Such an increase in sugar content also explains the slight but definitely increased yield of butyl alcohol from sprouted corn, since the diastatic enzymes of the corn germ attack the starch during germination. In none of these fermentations, however, was the yield of butyl alcohol compar-

able to that obtained from potato. Furthermore, the addition of a water extract of potato, either crude or purified, enabled the organism to ferment corn as vigorously as it did potato.

Preparation and purification of potato extract. Since the active principle in potato was found in a water extract, large quantities of crude extract were prepared for future use by the following procedure. The washed raw potatoes were ground, the juice was pressed out, the residue was washed twice with distilled water and pressed. The juice and washings were united, the starch was allowed to settle, and the clear supernatant liquid was siphoned off

TABLE 2

Effect of various supplements on production of solvents from corn by culture 21
(Calculated for 100 grams dry matter)

ADDITIONS TO 100 cc. 4 PER CENT CORN MASH	BUTYL ALCOHOL	ETHYL ALCOHOL	ACETONE	TOTAL SOLVENTS
	grams	grams	grams	grams
None	0 14	0 97	0 98	2 09
1 gram peptone	3 18	0 89	1 74	5 81
2 grams diastase (no digestion)	0 23	1 28	0 72	2 23
2 grams diastase (digested)	4 83	0 12	0 65	5 60
Sprouted corn used	0 86	0 99	1 73	3 48
21 cc. crude potato extract*	14 30	0 60	1 09	16 99
6 cc. purified potato extract*	9 60	0 43	0 96	10 99
15 cc. purified potato extract*	11 70	0 17	1 18	13 05
30 cc. purified potato extract*	14 42	0 22	0 95	15 59

* One cubic centimeter of extract represents 1.0 gram raw potato.

and filtered. The solution was then heated to precipitate heat-coagulable proteins, and these were filtered off. The filtrate was sterilized in flasks and stored until needed. Since this crude extract was found to contain starch, sugar and protein, it was purified by precipitating these substances. An excess of ammonium hydroxide and about 30 cc. of a saturated solution of lead acetate were added to each 100 cc. of crude extract. The solution was filtered several times in a Büchner funnel through Norit. The excess ammonia was then boiled off, and the lead removed with H_2S . This procedure was found to remove all detectable traces of starch (iodine test), sugar and substances hydrolyzable to sugar

(Fehling's test), and proteins (biuret test). As may be seen from table 2, the extract is practically as stimulating after this treatment as before.

Effect of other vegetable extracts on the fermentation of corn. The occurrence of the stimulant in plant materials other than potato was determined by testing extracts from such materials prepared in the same way as the potato extract. Dry materials, such as peas and beans, were first soaked in water, pressed and treated in the usual way. Concentrated corn-steep which was obtained from the Corn Products Refining Company was also purified by the lead acetate, etc., treatment. The extracts were tested for their stimulatory action on the fermentation of corn by culture 21. The curves in figure 1 show the stimulative effect of some of these extracts as measured by butyl alcohol production. All the extracts were found to contain the stimulant, but in varying concentrations. For example, a degree of stimulation which required 9 cc. of malt-sprout extract could be brought about by 1.5 cc. of pea or potato extract. Corn-steep is one of the materials richest in the stimulant. This may seem contradictory in view of the failure of corn mash alone to undergo a butyl fermentation. This apparent anomaly may be explained by the concentration of the factor in the preparation of the corn-steep. In corn meal, while probably not absent, the factor is present in too small a quantity to bring about any considerable fermentation of starch. It should be noticed that all the curves tend to flatten out after the maximum butyl-alcohol production is reached, which maximum is nearly the same in all cases. This shows that the effect is proportional to the concentration of the stimulant up to a certain point, but that thereafter, a higher concentration has no effect.

Certain other plant materials contained the stimulant to a more marked degree than those shown in figure 1. The curves representing the effects of extracts of cabbage, orange and lettuce are shown in figure 2. The much greater concentration of the stimulant in these materials (on the dry weight basis) made it necessary to modify the horizontal scale on the figure. The general shape of the curves is very similar to those in figure 1. In the case of the lettuce extract the concentration of ammonium acetate due to the

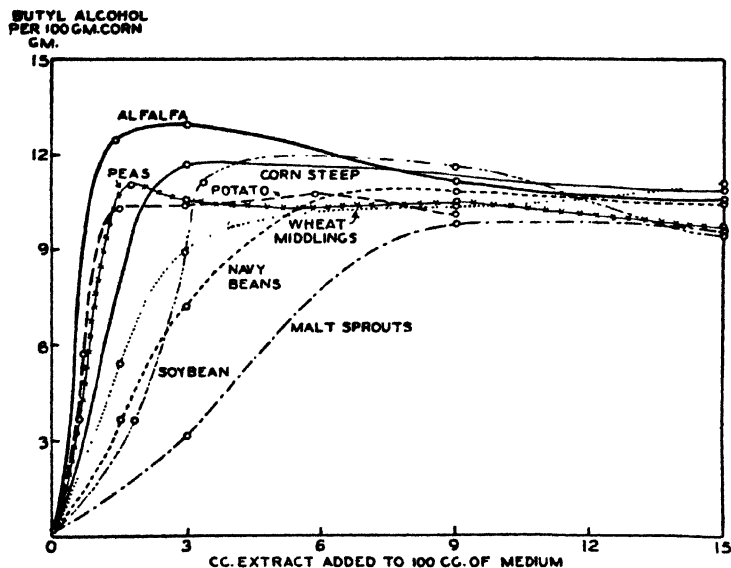


FIG. 1. EFFECT OF PLANT EXTRACTS ON FERMENTATION OF CORN BY CULTURE 21
(Excepting corn-steep 1 cc. is equivalent to 1 gram dry matter)

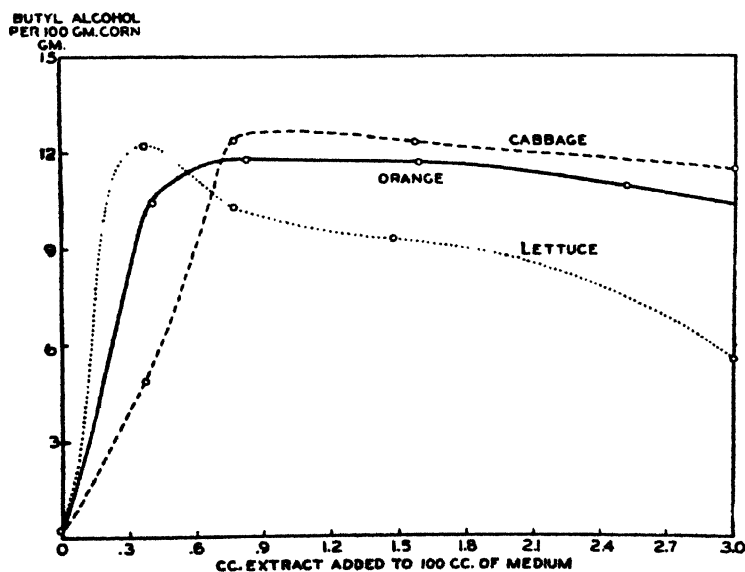


FIG. 2. EFFECT OF CABBAGE, ORANGE AND LETTUCE EXTRACTS ON FERMENTATION
OF CORN BY CULTURE 21
(One cubic centimeter is equivalent to 1 gram dry matter)

method of preparation was so large that it became toxic in the larger amounts added. This explains the dropping off in the curve. Lettuce, cabbage and orange seem to contain from five to ten times as much stimulant per unit dry weight as the other sub-

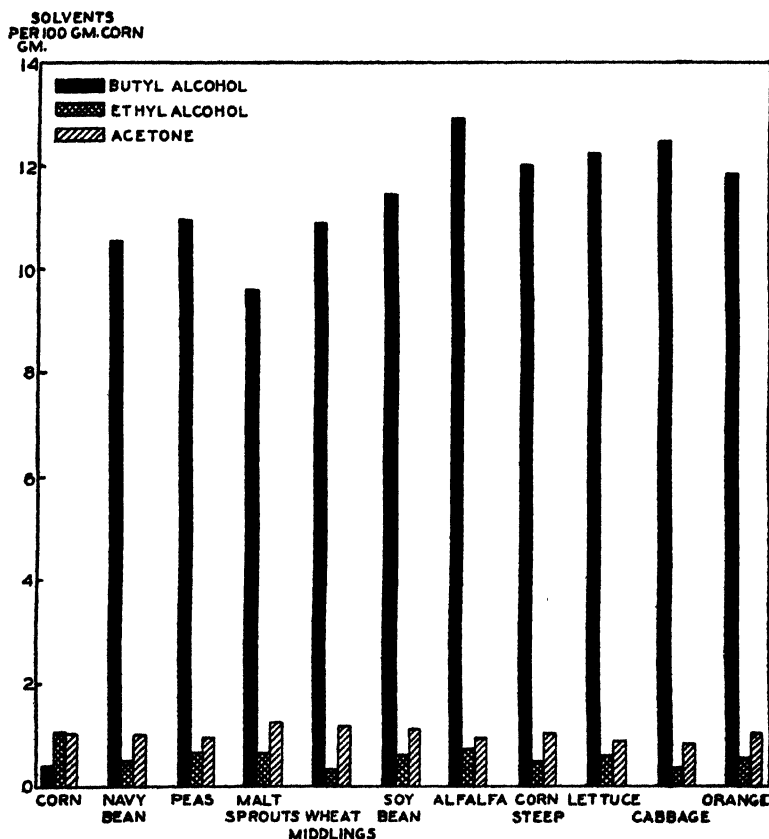


FIG. 3. EFFECT OF PLANT EXTRACTS ON PRODUCTS FORMED FROM CORN BY CULTURE 21

stances tried. An idea of the relative concentration of stimulant in these substances may be obtained from the slope of the curves from the control point to the flat portion. Lettuce seems to be the most concentrated source of the stimulant, with orange and cabbage nearly as concentrated. Alfalfa seems to be the best of

the other substances, and malt sprouts apparently is the poorest. The effect of the various plant extracts on the yield of all solvents by culture 21 is given in figure 3. The data are given only for the point of maximum stimulation. As was indicated previously (table 1) the only one of the three solvents to show any appreciable increase was butyl alcohol.

In order to determine whether these extracts stimulated other butyric acid bacteria besides culture 21, soy-bean, pea and corn-

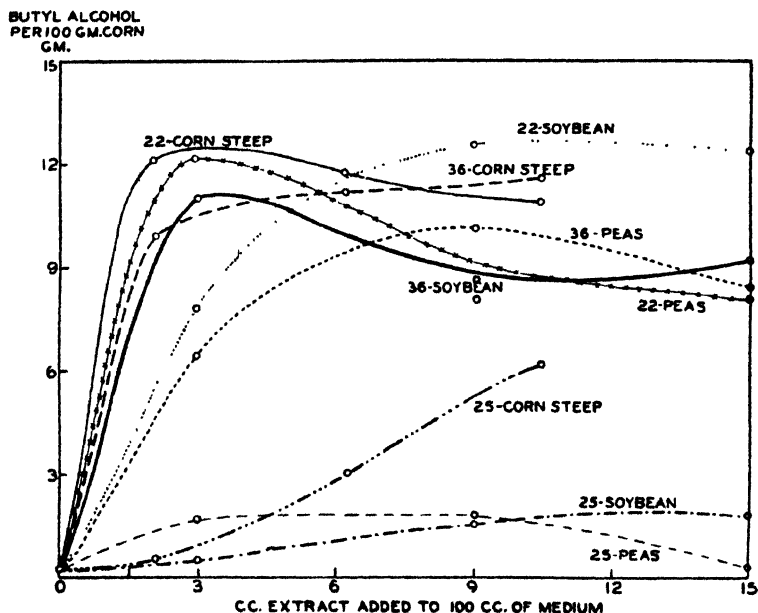


FIG. 4. EFFECT OF PLANT EXTRACTS ON FERMENTATION OF CORN BY CULTURES 22, 25 AND 36

(Excepting corn-steep 1 cc. is equivalent to 1 gram dry matter)

steep extract were added to corn and fermented with several other strains of butyric acid bacteria. The curves in figure 4 show the effect of the extracts on these organisms. Culture 25 was very slightly affected by the stimulant, while cultures 22 and 36 showed a response quite similar to that of culture 21. Attention is called to the fact that these organisms exhibited the same differences in fermentation when grown on potato (table 1). Figure 5 summa-

rizes the data on the solvent production of these organisms at the points of maximum yields.

It should be noted that the solvent production by the three strains showing marked stimulation, (i.e. cultures 21, 22 and

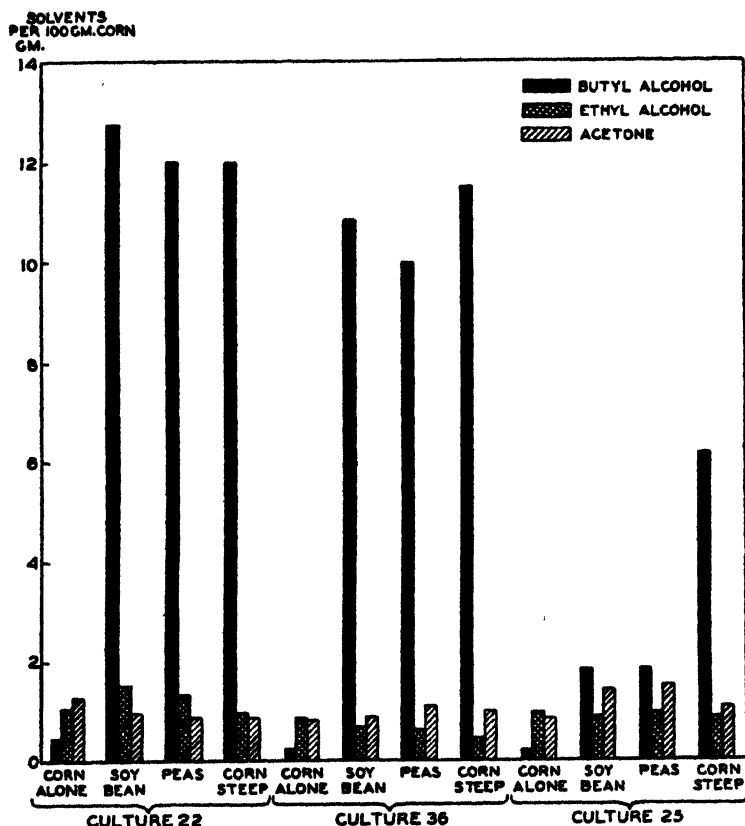


FIG. 5. EFFECT OF PLANT EXTRACTS ON PRODUCTS FORMED FROM CORN BY CULTURES 22, 25 AND 36

36) is of about the same magnitude, and that this same yield is obtained irrespective of the source of the stimulant (figs. 3 and 5). It seems logical to assume that the same factor is concerned in all cases, since it is improbable that there would be more than one factor which would resist the treatment involved in the prepara-

tion of the extracts (heating, filtration through Norit, treatment with ammoniacal lead acetate, and precipitation of lead sulfide) and which would at the same time affect different butyric acid bacteria in an identical manner.

SUMMARY

An unknown substance which greatly stimulates the fermentation of corn-mash by certain butyric acid bacteria has been found in potatoes, yams, oranges, lettuce, cabbage, alfalfa, soy- and navy beans, wheat middlings and malt sprouts. This substance appears to be low or lacking in corn, rice, oats and barley.

The effect of the unknown stimulant is greatly to increase the destruction of starch and to increase the production of butyl alcohol more than tenfold. Yields of other solvents (acetone and ethyl alcohol) are not affected.

While many plant materials contain the stimulant, the concentration varies. On the basis of dry matter, lettuce, cabbage and orange contain from five to ten times as much stimulant as the other plant materials tested.

Different strains of the butyric acid bacteria have been shown to differ widely in the degree of response to the stimulant.

A method of preparing extracts of vegetable materials involving purification with ammoniacal lead acetate is described. Such extracts are free from detectable traces of glucose, carbohydrates hydrolyzable to glucose, and proteins.

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IRRADIATION OF PLANT VIRUSES AND OF MICRO-ORGANISMS WITH MONOCHROMATIC LIGHT¹

I. THE VIRUS OF TYPICAL TOBACCO MOSAIC AND SERRATIA MARCESCENS AS INFLUENCED BY ULTRAVIOLET AND VISIBLE LIGHT

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The effects of visible light and, in recent years more especially, the bactericidal effects of ultraviolet light have received increasing attention. Most of those engaged upon such problems prior to very recent years have been content with results essentially qualitative in nature. Nevertheless, progress was made and it was to be expected that with the development of vastly improved physical instruments available to the biologist, and with analogous advances in biological technique, definitely quantitative studies would be made. This advance is now in progress and during the last five years, especially, contributions of much fundamental importance have appeared. Such studies are of interest not merely because they afford definite data regarding the lethal effect, or lack of lethal effect, of monochromatic light of various wave-lengths upon bacteria, but also because they indicate the rate of killing, or diminishing rate of survival with time and with increasing intensity of illumination. Prominent among those who have contributed these recent advances may be mentioned Bayne-Jones and Van der Lingen (1923), Coblentz and Fulton (1924), Gates (1929, '29a, '30), Wyckoff (1932), and Ehrismann and Noethling (1932). Virus and bacteriophage have

¹ This work was made possible, in part, through a grant from the Research Committee, Graduate School, University of Wisconsin.

² In part this represents work done while holding a National Research Fellowship in the Biological Sciences.

received far less attention. Nevertheless, progress has been made, notably through the contributions of McKinley, Fisher, and Holden (1926), Fisher and McKinley (1927), Olitsky and Gates (1927), Rivers and Gates (1928), and Baker and Nana-vutty (1929). Obviously more quantitative data on the effect of radiation on viruses are most desirable, and in many cases at least are experimentally obtainable. The ease of handling plant viruses at once suggests the desirability of working upon some one or more of these forms. This study was undertaken with primary interest for the moment centered upon the virus. *Serratia marcescens* was selected as a species of bacteria suitable for comparative study.

The purpose of this paper is accordingly to report the technique employed and also the results obtained with the influence of approximately pure monochromatic light, primarily ultraviolet, on a plant virus, the agency of typical mosaic of tobacco, in comparison with the vegetative phase of a species of bacteria, carried out under conditions which are believed to be as nearly quantitative as is practicable with standard procedures and available apparatus.

Although the amount of work done with the bacteria has been extensive, such studies have been primarily for comparison and control. Were it not necessary to make this comparison as nearly absolute as possible, it would be entirely feasible to approach by new and improved technique a still more closely quantitative determination of the influences of radiation of different wave lengths on the bacteria. Such a study is in progress.

A fundamental difficulty which underlies the utilization of any virus as an object to be irradiated is found in the fact that viruses do not lend themselves as yet to methods of intensive purification without great possibility of loss of activity. In a final analysis of the influence of light of any wave-length it would be desirable, of course, to eliminate from the virus suspension all other particles or substances which might absorb light. Moreover, there is no way in which the extent of this constant error of inefficient absorption can be accurately determined. Unfortunately, in the past investigators have made the exposures of viruses

primarily in the virus-containing fluids, and frequently have made comparisons between these results and the results obtained with bacteria or other organisms exposed under entirely different conditions—usually on agar plates. The agar plate method is obviously unsatisfactory for virus work, and it possesses disadvantages as applied to bacteria. In the studies here reported a development of the suspension technique has been found peculiarly applicable for both materials, as indicated later.

PREPARATION OF THE BIOLOGICAL MATERIALS

In our work we have purified the virus of typical tobacco mosaic up to a certain limit by using a diatomaceous earth adsorbent and precipitant. This material eliminates all chlorophyll and much of the proteinaceous materials, likewise much of the complex carbohydrates of the leaf juices, yet leaves the plant extract essentially in a "natural" condition with respect to solutes. The virus suspension obtained still contains a small amount of some soluble pigmented substances, possibly flavones and some tannoid complexes.³ The difficulty of making an accurate comparison between the tolerance of the virus and of the bacteria, determined separately, was recognized, since the ineffective light absorption of the two suspensions would introduce errors not readily computed.

The difficulty referred to above was eventually very simply solved by diffusing the bacteria in the virus suspension and thus exposing both the bacteria and the virus agency under identical conditions. The combination of virus and bacteria in the suspension, under the conditions maintained, affects neither material deleteriously, so far as can be measured. Such technique would

³ Fresh, succulent, diseased tobacco shoots are finely ground in a food chopper; the juice is then squeezed through gauze, diluted by the addition of nine parts of water to one of juice, giving a 1:10 suspension of the virus. To 100 cc. of this standard 1:10 suspension is added 10 grams of super celite (a diatomaceous earth) and after standing (with occasional agitation), the material is centrifuged at approximately 4000 r.p.m. for five to ten minutes, and the supernatant, clear, slightly colored suspension is used as a source of virus. Subsequently, as noted later, this suspension is again diluted to a concentration, relative to the natural juice of 10^{-2} , here designated as 1:100 suspension.

at least furnish comparable results. This was not accomplished, however, without considerable experimental work and the introduction of several other modifications of the usual procedure. In the first place, when the 1:10 virus-containing plant juice is prepared as described, some of the bacteria accidentally present on the leaves may be found in the juice, nor does the "precipitation" by means of diatomaceous earth remove all of these. It was then found perfectly practicable to pasteurize the 1:10 virus suspension by exposure in a water bath, with stirring, at a temperature of 65 to 67°C. for fifteen minutes. Such spores of bacteria as may remain in living condition are not a factor of importance in view both of the dilution to 1:100 of the juice for exposure to radiation, and of a further 100-fold dilution when the bacterial plates are poured, as subsequently indicated.

Moreover, very early during our preliminary work it was clear that the bacteria used would not form a perfect suspension either in distilled water or in the diluted virus suspension. Clumping occurred and this gave rise, of course, to erratic results when the bacteria were poured on the dilution plate. Eventually the difficulty of clumping was entirely prevented by the utilization of a physiological salt solution, that is, a balanced salt solution containing the chlorides of calcium, sodium, and potassium.⁴ It was found less desirable to use sodium chloride alone.

Accordingly our procedure was to prepare a virus suspension in the usual way; likewise to arrange by a definite culture system for a fresh culture of the bacteria which, under the conditions of growth and time interval, would furnish a rather definite number of organisms per cubic centimeter. From an agar slant culture of the bacteria, grown for twenty-four hours at approximately 28°C., a suspension of standard opacity was prepared. Tubes of standard beef bouillon were inoculated from this suspension, each receiving 1 cc., and the broth cultures were incubated for eight

⁴ This salt solution was of the following composition:

NaCl.....	6 grams
KCl.....	0.4 gram
CaCl ₂	0.4 gram
Water.....	1,000 cc.

hours at 28°C. These tubes were used immediately as stock sources of bacteria, or, if any short delay occurred, they were plunged into crushed ice. As a matter of practice we arranged that the stock solutions of the virus suspension would be one-tenth the concentration of the virus in the plant juices and the stock bacterial suspension would contain approximately 100,000,000 bacteria per cubic centimeter. These two stock suspensions were then added to the physiological salt solution in such quantity that the final suspension of virus was 1:100 and the final suspension of bacteria of the order of 1,000,000 per cubic centimeter.

From the preceding statements it will be seen that the suspension for exposure actually contained in 100 cc. of the salt solution approximately 1 cc. of bouillon containing the bacteria and what amounts to 1 cc. of the virus suspension calculated on the basis of the full-strength juice. The ineffective absorption of the final suspension is, of course, to be ascribed chiefly to the addition of the virus fluid, though in part also to the bouillon added. This exposure suspension was then kept in an ice mixture until used.

APPARATUS

For a quantitative study of the effect of radiation it is desirable to work with light as nearly monochromatic as possible and an accurate determination must be made of the amounts of energy used. It is with monochromatic light alone that it is possible to determine effects such as the localization of the influence of light in the different parts of the spectrum; while exact energy determinations are required for a quantitative interpretation of the results.

It appeared early in our investigation that the energy necessary to inactivate the virus agent with the suspension method of exposure decided upon is unusually large, very much larger, in fact, than the energies necessary to kill microorganisms, also larger than the energies usually available in work with truly monochromatic light.

The use of filters which are now available for the differential selection or elimination of certain parts of the spectrum would at

once suggest itself. However, while the loss of energy with the use of such filters may be only moderate, they show certain other disadvantages which render them unsuitable for our work. The best filters available will transmit relatively broad regions of the spectrum and the band transmitted will not show a definite cut-off. If practicable, it would have been desirable to work with a double monochromator by means of which very pure monochromatic light might be obtained. This possibility was precluded by the fact that the energies needed in this experiment were very much larger than could be obtained with a double monochromator, so that we had to compromise and be satisfied with the use of a single monochromator with very high transmitting power. There was available for part of our work, fortunately, a monochromator with very large fused quartz optics and a source of light considerably more intense than the commercial mercury vapor lamp.

Source of radiation. The greater part of this investigation was carried out with a water-cooled, capillary, mercury-vapor lamp of the Daniels-Heidt (1932) type, burning on 150 to 400 volts and using 5 to 2 amperes. The high intensity of the radiation (about 20 times greater per unit area of the arc than that of commercial lamps) made this lamp very suitable for our work.

Monochromators. Most of the work here reported was done with a large quartz monochromator consisting of a 60° fused quartz prism, with faces 12 by 14 cm., and a pair of plano-convex quartz lenses each with a diameter of 15.25 cm. and a focal length of 35 cm. (Heidt and Daniels, 1932). This instrument was used only down to $\lambda 2650\text{\AA}$, on account of the absorption of the fused quartz at shorter wave lengths. In connection with the instrument just described, there was available a large surface thermopile⁵ of 48 copper-constantan junctions in connection with a Leeds and Northrup galvanometer, H. S. type, the sensitivity of this set-up being about 170 ergs per second per centimeter deflection.

⁵ It is expected that a complete description of this thermopile, as well as of the thermopile and monochromator described in the following paragraph, will be given by Daniels and associates in an article to appear in the Journal of the American Chemical Society.

The entire work in the visible part of the spectrum was done on a monochromator of the constant deviation type, consisting of a 60° hollow glass prism (sides 12 cm. square) filled with ethyl cinnamate, and a pair of biconvex glass lenses each with a diameter of 12 cm. and focal length of 18 cm. This instrument was equipped with a large surface thermopile of 13 copper-constantan junctions, connected with a Leeds and Northrup H. S. type galvanometer, sensitivity 165 ergs per second per centimeter deflection. This, as well as the previously described monochromator, was used only with the capillary mercury vapor lamp.

Both of the instruments described in the paragraphs on source of radiation and monochromators were kindly put at our disposal by Professor Farrington Daniels of the Department of Physical Chemistry.

A Bausch and Lomb constant-deviation type, quartz monochromator in connections with a vacuum thermopile and a Kipp and Zoonen galvanometer was used in some of the work. These instruments were kindly put at our disposal by Professor C. E. Mendenhall of the Department of Physics.

The work on the 2537Å line and the checking work on the other lines of the spectrum were done with an apparatus built around a Bausch and Lomb monochromator loaned through the Radiation Committee of the Division of Biology and Agriculture, National Research Council. A description of this experimental set-up will be published shortly.

In the following list are given in Ångström units the wave lengths employed; in certain cases it was necessary to group certain lines together, and in such cases an approximate designation is given, the actual lines being given in parentheses: 2537 (2535, 2537), 2652 (2652, 2653), 2804, 2952 (2925, 2967, 3021, 3023), 3130 (3125, 3131, 3132), 3342, 3650 (3650, 3654, 3662, 3663), 4047 (4047, 4078, 4108), 4358 (4339, 4347, 4358), 5461, 5790 (5770, 5791), 6120 (6072, 6123).

The purity of the lines was tested in many cases with a quartz spectrograph. The spectrum showed that there was never more than 10 per cent, and in some cases not even 2 per cent, of light of other wave-lengths present.

Exposure vessels. (a) The virus-bacterial suspension already described was consistently irradiated, for the greater part of this investigation, in a standard fused quartz cell (fig. 1). The dimensions of this exposure vessel were as follows: height 11.5 cm., width 1.5 cm. The faces of the cell were parallel, and these were ground and polished up to a height of 5.5 cm. The throat

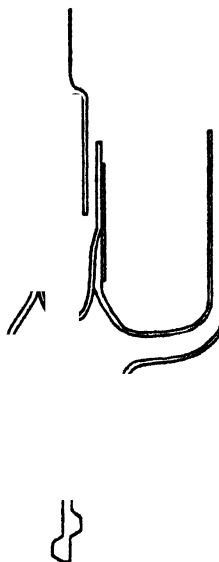


FIG. 1. QUARTZ EXPOSURE-CELL, WITH STIRRER AND LIQUID SEAL

of the cell was cylindrical and provided with a ground mouth into which fitted a ground stopper through which was introduced a quartz stirrer reaching well into the liquid to be exposed. The end of the stirrer was so constructed that when turning it would not only mix the liquid by rotation but would also lift the material and thus guarantee perfect circulation. Care was taken that the stirrer should make at least 300 revolutions per minute, and no exposure-interval was started until the material had been in

motion for two to three minutes. Moreover, the location of the stem of the stirrer was so arranged that it was not in the path of the beam of incident light.⁶ This cell was used for all work down to $\lambda 2652\text{\AA}$. The absorption of the cell down to this line was negligible. The work at $\lambda 2537\text{\AA}$ and the checking of the effects at other lines were done in a cell of novel design, with crystalline quartz sides, which will be described in a later paper.

Experimental procedure. The cell and stirrer were cleaned with the concentrated sulphuric acid-dichromate cleaning solution

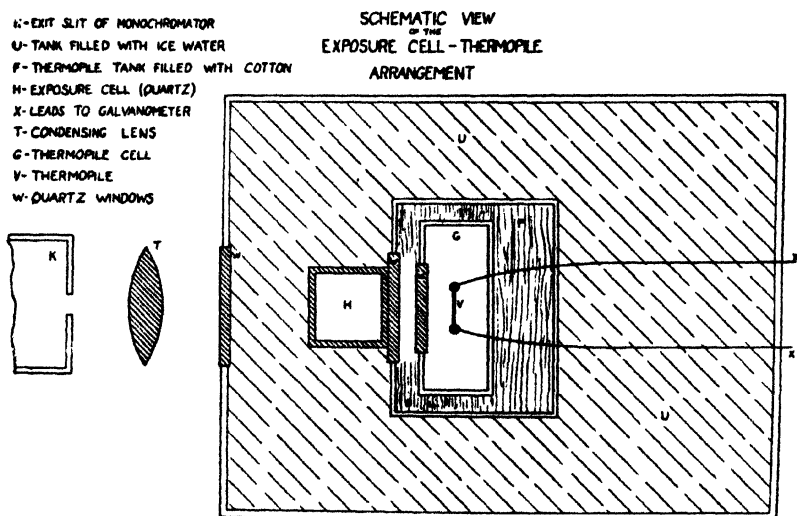


FIG. 2. EXPOSURE-TANK, CELL, AND THERMOPILE ARRANGEMENT

and they were then washed at least 10 times with distilled water and at least twice with sterile distilled water. The cell was filled with distilled water and placed at H in front of the thermopile in the tank containing ice water, the entire set-up being obvious from the illustration (see fig. 2). The calculation proceeded as follows: First, there were measured the values of the energy transmitted by the cell filled with water; secondly, values were

⁶ Mr. W. H. Bauer of the Department of Physical Chemistry did the quartz blowing required, assisted in some of the apparatus set-ups, and in some of the exposures of material.

obtained (1) by measuring the energy transmitted by the cell filled with the standard suspension, (2) subtracting such values from corresponding values of the water-filled cell, (3) dividing this result by the number of cubic centimeters of suspension in the cell, and (4) multiplying this quotient by the magnitude (in seconds) of the exposure-period.

Controls, employed with every experiment, were handled in a manner identical with those of the exposed, except for the complete protection from irradiation. The absorption of the different batches of exposure-materials as prepared for the several experimental series varies somewhat. These variations are ascribable to the virus suspension, that is, to the influence of the environmental conditions under which the diseased plants are grown on the color of the plant juice. Since the virus material before exposure had been diluted to 1:100, small changes were not very obvious in the absorption spectrum. If the purified and undiluted virus suspension exhibited too much color, it was usually discarded and a new lot of fresh material prepared. Even small changes in the absorption spectrum of the material to be investigated for the influence of monochromatic light are not ideal, even though the material may appear clear and colorless to the untrained eye. So long as there are no better methods for purifying the virus suspension we had to be satisfied.

In order to make the results as comparable as possible, each set of experiments was run with one batch of exposure-suspension, kept in crushed ice during the entire interval. The absorption of the material (stored in an ice water bath) did not change in sixty hours sufficiently to appear in the absorption readings. For best results the entire spectrum was investigated from one batch of material, this requiring a continuous sequence of exposures for a period as long as sixty-five hours.

TREATMENT OF SUSPENSIONS AFTER EXPOSURE AND DETERMINATION OF LETHAL EFFECTS

After exposure, the irradiated suspensions and the control material were pipetted into sterile test tubes and the plugged tubes held in crushed ice. From each tube of the exposed sus-

pension (representing one interval of exposure) and of the control, unirradiated suspension, there were prepared two series of inocula, the one to be used in making a dilution series of poured plates for bacterial counts and the other for inoculation of tobacco plants as a means of determining the extent, if any, of virus inactivation.

Poured plates and counting the bacteria. The "original" of the exposure-dilution and of the unirradiated control, each containing around 1,000,000 bacteria per cubic centimeter, of each exposure interval, was designated No. 1. From each No. 1, as soon as removed from the ice water bath, a series of dilutions was accurately and aseptically prepared in the physiological salt solution previously described, and corresponding agar plates were poured.

In order to insure the highest accuracy in the bacterial counts the greatest care must be taken in mixing or shaking, adequate shaking being given in every case prior to removing a pipette sample either for further dilution or for transfer to the corresponding Petri dish. Ordinarily we used dilutions Nos. 3, 4, 5, and 6. Transfers of 1 cc. each were accordingly made to a duplicate series of Petri dishes, and the agar poured, as usual, at 43 to 45°C. Standard potato glucose agar was used, at pH 6.5 to 7, although the organism grows well on various nutrient agars.

Serratia marcescens grows rapidly at 24 to 28°C., with readily identifiable colonies, and with little spreading of the surface colonies if the agar is moderately hard, so that the counts were made ordinarily after forty-eight to twenty-four hours, the entire plate being carefully counted.

Since a control series was arranged for each of the longer exposures and one control for each of two of the shorter exposures, the lethal effect was directly determined as the percentage reduction in the number of colonies in the exposed compared with the control. It appears that this dilution plate count method is as quantitative as it seems practicable to apply in bacteriological work. Nevertheless, exact knowledge of the behavior of the experimental organism is a necessary preliminary to careful work.

Inoculation with the virus. Since the only available method of determining inactivation of this virus is by the decrease in the

incidence of disease induced when the virus is inoculated into a susceptible host, it was necessary to use such a host, yielding characteristic symptoms. An established strain (Wisconsin Havana 142) of cultivated tobacco was selected and grown in pots in the greenhouse for this purpose. For each exposure and similarly for each control a lot of not less than twenty plants was employed, ten plants of each lot being inoculated at the exposure-dilution (1:100) of the virus and ten plants at greater dilution (1:1000). The latter dilution usually affords, with a standard needle-prick method of inoculation 100 or 90 per cent of disease. At a dilution of 1:100 it is seldom that inoculation fails to yield 100 per cent infection, although during extreme weather infection may be erratic, and under unfavorable growth conditions the symptoms may be temporarily masked.

It is obvious that since the number of plants practically available for inoculation must be limited, the degree of accuracy to be expected in the virus work cannot approach that attainable with the bacteria used for comparison. There is also the inoculation factor to be considered. We have invariably repeated any tests made during midsummer, or those otherwise demanding confirmation. Nevertheless, it is to be recognized that the values found for the virus agent are reproducible only within the limits of the error of inoculation.

RESULTS AND DISCUSSION

To illustrate the results obtained in respect to the lethal action of the irradiation, a table for one set of experiments, $\lambda 2652\text{\AA}$, is given (table 1). This table exhibits under the heading "incident energy" the amount of energy which strikes the exposed material; and under "absorbed" is given the energy which is lost by the beam in passing through the exposure-cell and before reaching the thermopile. These values are corrected for the absorption and reflection of the windows, the latter being the largest source of error when not taken into account. In addition, there are two factors which modify very slightly the values given, and these will not be evaluated in this paper. The factors referred to are (1) the light scattered by the material exposed, and (2) the ex-

hibition by the exposure-material of a faint bluish white fluorescence. In further explanation of the first factor it may be said that the virus particles (or the material carrying the virus), the cells of the bacteria, and such other particles as are furnished by the very dilute bouillon and virus fluids are all jointly concerned. The factor of fluorescence is an extremely small one and is usually

TABLE 1

Radiation of virus-bacterial suspension, at $\lambda 2652 \text{ \AA}$, employing a monochromator with fused quartz optics and a capillary mercury vapor lamp as the source of radiation

TIME OF EXPOSURE	ENERGY		LETHAL EFFECTS		
	Incident	Absorbed	Bacteria killed	Virus inactivated	
				1:100*	1:1000†
<i>seconds</i> $\times 10^3$	<i>ergs</i> $\times 10^3$ per cc.	<i>ergs</i> $\times 10^3$ per cc.	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0.06	12.82	9.6	70		
0.16	36.12	27.2	93		
0.20	46.38	34.78	99.7		
0.23	54.93	41.2	99.98		
0.26	64.59	48.4	100		
0.30	53.8	40.4	100	0	0
0.60	112.4	84.3	100	0	0
1.2	225.32	181.9	100	0	0
1.8	367.12	289	100	0	0
2.4	530.62	412	100	0	0
1.2	734	551	100	0	60
2.4	1,468	1,102	100	20	60
4.8	2,936	2,204	100	30	90
7.2	4,404	3,306	100	60	100
10.8	6,606	4,959	100	100	100

* This being the exposure dilution, the same dilution was also used for inoculation.

† Exposure-dilution was diluted to 1:1000 for inoculation.

ignored. Furthermore, while we consider that our results approach absolute values, they are primarily intended to be quantitatively comparative. In particular relation to this last statement, it should be noted that even though we worked with a considerable number of wave-lengths, actually the effective spectral range, or range of characteristically strong lethal effect (within the limits investigated by us), is confined to a very narrow region

of the spectrum; and since the difference in the amount of scattering relative to wave-length is small, it can be wholly ignored in this comparative study.

Regarding the effect of wave-length on the virus, our results indicate that lethal action (inactivation) is confined to wave-lengths shorter than about 3100\AA . At approximately this wave-length about 100 times as much energy was used as at $\lambda 2652\text{\AA}$. At such high values, there was probably some scattered light. It will be noticed that the energy values representing 100 per

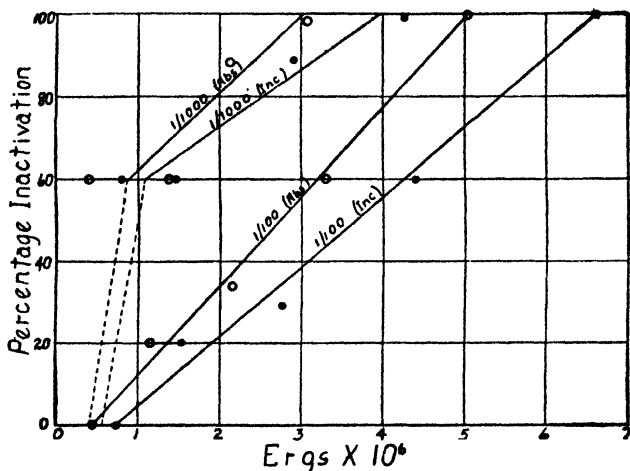


FIG. 3. PERCENTAGE INACTIVATION OF TOBACCO VIRUS, EXPOSED AT 1:100 DILUTION, DILUTION OF 1:1000 MADE AFTER EXPOSURE AT $\lambda 2652\text{\AA}$

cent killing of the bacteria are far below the values having any measurable influence on the virus.

With the bacteria lethal effects are also primarily confined to wave-lengths shorter than about 3100\AA , but we are not yet prepared to say that there are no effects at longer wave-lengths. In fact, with energy 50,000 times what was needed to give killing effects at $\lambda 2652\text{\AA}$ we were able to show, with *Serratia marcescens*, some killing effect in certain wave-lengths of the visible spectrum, in spite of the fact that we took all practicable precautions to exclude ultraviolet light, using a monochromator with glass optics, a liquid (carbon bisulphide) prism, and in addition several filters

of window glass. It is proposed to investigate further this particular relation, that is, wave-lengths in the range of the visible.

As presented in figure 3 the graphs show, in a typical manner, the lethal effect of the irradiation of the virus at $\lambda 2652\text{\AA}$. Attention is drawn particularly to the virus behavior at concentration 1:100. Representing this concentration two curves are given for percentage killing effects, the one curve displaying incident energy, and the other the energy absorbed. At virus concentration 1:100 the results, as would be expected, are less subject to error through the inoculation procedure. There are also presented in the same figure corresponding curves for the virus dilution 1:1000, and here, as previously indicated, the reliability of the results is somewhat lessened. Moreover, variability in the results is always slightly intensified with increasing lethal effect of the wave-length, so that at $\lambda 2652\text{\AA}$ we obtain our greatest divergencies.

In the case of the virus the middle part of the curves invariably offers the greater satisfaction. This, however, would be true whether dealing with the lethal effects of irradiation, of temperature, or of toxic agents. On the one hand, a slight effect means one or two healthy plants in a lot of ten or twenty; whereas a pronounced inactivation of the virus means one to two diseased plants in a similar lot. Intermediate effects obviously furnish a more reasonable number of plants in each class, and accordingly better values are obtained.

The same type of curve displayed in figure 3 has been obtained for the various wave-lengths investigated with which lethal effects were obtained. These curves are not presented in detail, but smoothed curves for the virus at 1:100 and 1:1000 are given in figure 4, exhibiting the incident energy employed. In order to present clearly the results, points taken from the curves in figure 4 above are used in figure 5 to portray for the virus the behavior towards wave-lengths in relation to 25, 50, and 75 per cent inactivation. In the same figure are given the killing effects with *Serratia marcescens* of the various wave-lengths—based on the determinations in the presence of the virus—and using points

corresponding to 70 per cent killing. It will be seen that maximum inactivation occurs at $\lambda 2652 \text{ \AA}$.

Attention is drawn to the dotted lines for $\lambda 2537 \text{ \AA}$, which were obtained under somewhat different conditions, and accordingly are represented here only tentatively. The relation to this wave-length will be treated more in detail in a subsequent paper.

Contrasting the radiation results as between bacteria and virus, the energies required at the effective wave-lengths to produce equivalent lethal effects when the agents are irradiated coin-

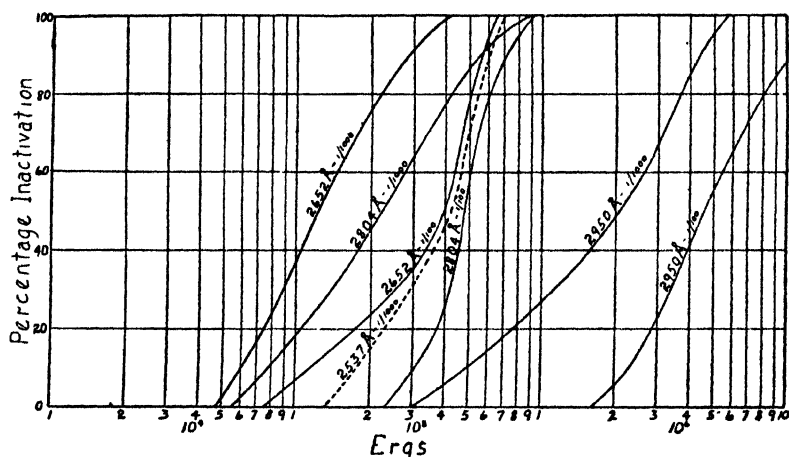


FIG. 4. INACTIVATION OF TOBACCO VIRUS AT ALL EFFECTIVE WAVE-LENGTHS

ciently in the same suspension are 200 times as much for the virus as for the bacteria.

For a quantitative interpretation of the results from the standpoint of the work with the virus, it is desirable to compare the absorption coefficients at the several wave-lengths with the corresponding lethal effects of these wave-lengths. The curves representing these values show a close agreement down to $\lambda 2652 \text{ \AA}$. At $\lambda 2537 \text{ \AA}$, the lethal effect again decreases, while the absorption coefficient increases very rapidly, this increase being probably related to the coloring matters present, as previously indicated. Accordingly, this does not introduce as large an error as might at first appear. Incidentally it will be observed

that the values reported relative to wave-length are consistent for the bacteria, for example, with values which have been reported where the bacteria alone were irradiated, as by Gates (1929), and by Ehrismann and Noethling (1932).

Results of very different types have been obtained by various investigators working primarily with certain animal viruses and the bacteriophage. Many of the investigations here referred to have been essentially general and qualitative. The apparatus

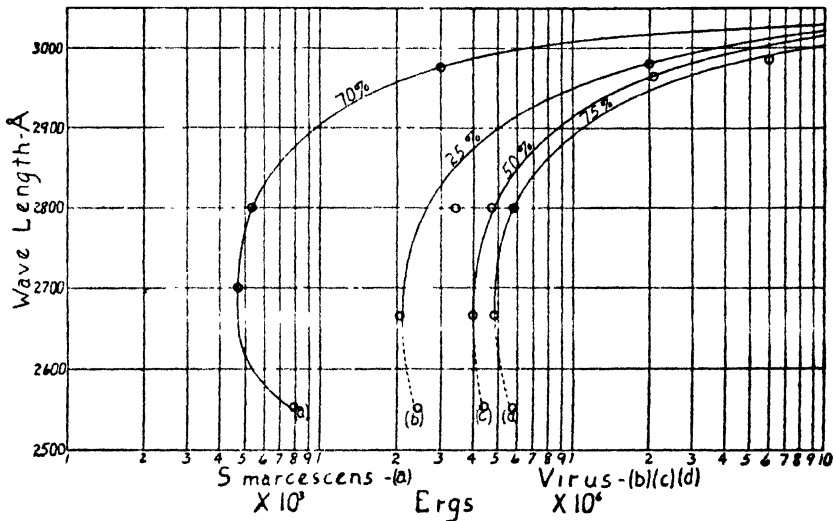


FIG. 5. TWENTY-FIVE, 50, AND 75 PER CENT INACTIVATION OF TOBACCO VIRUS AND 70 PER CENT KILLING EFFECT OF *SERRATIA MARCESCENS*
Note different energy scales

and techniques employed were not in those cases intended to determine the influence of monochromatic light, nor were measurements of intensities given. In certain instances killing effects with viruses were reported under conditions approximating those inducing bactericidal action. With respect to plant viruses, Mulvania (1926) and Smith (1926), working with leaf extracts containing the virus of tobacco mosaic, found inactivation of the virus when exposed to the full spectrum of the mercury arc. Using a purer preparation, Arthur and Newell (1928) found in-

activation of the same virus at wave-lengths shorter than $\lambda 3000$ Å. For the most part, however, the results do not warrant definite comparisons.

From results obtained by Olitsky and Gates (1927) it is apparent that under the influence of monochromatic radiation the energy values required to kill *Staphylococcus aureus* and to inactivate the virus of vesicular stomatitis are of the same order, and that $\lambda 2675$ Å is most effective for both virus and bacteria.

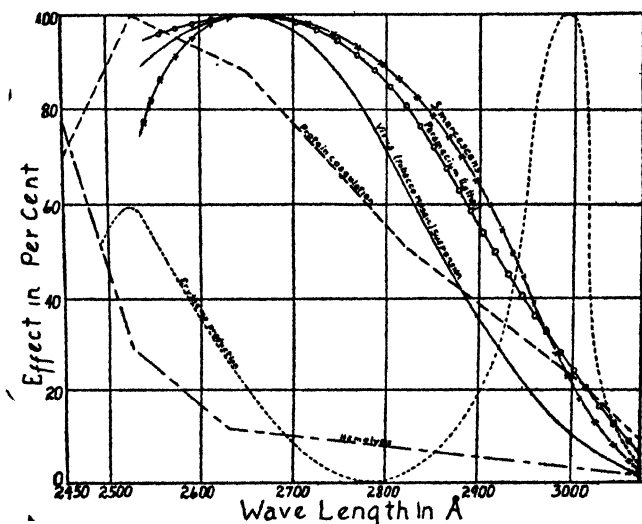


FIG. 6. RELATIVE BIOLOGICAL EFFECTS WITH RESPECT TO WAVE-LENGTH, IN THE ULTRAVIOLET BELOW $\lambda 3100$ Å, SELECTED FROM VARIOUS AUTHORS

(See text)

The materials were separately irradiated on the surface of agar plates. Similarly, Rivers and Gates (1928), using the same agar surface technique and monochromatic light, report that the curves of inactivation of vaccine virus approximate those exhibiting killing effect with *Staph. aureus*, the maximum killing effect being at $\lambda 2675$ Å. Baker and Peacock (1926) and Baker and Nanavutty (1929) employed a suspension method, diffusing both agents in the same saline, exposing in an open dish to the full spectrum of the mercury arc, and stirring the suspension merely by means of a jet of air. Their results indicate that the suscep-

tibility of the bacteriophage is of the same order as that of *B. coli*, while the resistance of the virus of Rous's chicken sarcoma is approximately five times as great as that of the bacteriophage.

In view of the fact that, considering relative energy values, the curves for bactericidal action and virus inactivation, relative to wave-length, are of the same general form, it is of interest to compare these curves with curves representing the results of other investigators using different biological materials. Accordingly, there are displayed in figure 6 graphs illustrating (in comparison with our data for virus and bacteria) protein coagulation, from Sonne (1928); lethal effect on paramecium, Weinstein (1930), erythema production, Hausser (1928) and Coblentz, Stair, and Hogue (1931), and hemolysis, Sonne (1928). It will be seen that the curves representing bactericidal action, virus inactivation, lethal effect on paramecium, and protein coagulation (see also Rivers and Gates, 1928) are of the same general type. This striking similarity will be discussed more at length in later publications.

SUMMARY

The physical installation used has included a quartz monochromator, an intense source of radiation (Daniels-Heidt capillary mercury vapor lamp), a quartz exposure-cell (mechanically stirred), an exposure tank provided with a quartz window, and a sensitive thermopile. Twelve spectral lines (or groups of lines) were investigated in the range $\lambda 2537-6120 \text{ \AA}$. The temperature of the exposure was maintained at 1 to 2°C. by means of melting ice.

The biological materials consisted of a fresh suspension of semi-purified tobacco virus and of cells of *S. marcescens* taken during the logarithmic growth phase from a bouillon culture. For the determination both of lethal effects on bacteria and of inactivation of the virus, materials were combined in the same suspension, so that comparative values might be obtained. Exposed materials were accompanied by controls similarly treated, except as to protection from radiation.

Dilutions of the bacteria were prepared in physiological salt

solution. Poured plates (agar) were made and the counts gave a quantitative comparison of the irradiated cultures with the unirradiated controls. The percentage inactivation of the virus was determined by inoculation of tobacco plants and by comparing the incidence of disease induced—exposed versus unirradiated.

Inactivation of the virus is confined to wave-lengths shorter than about $\lambda 3100\text{\AA}$. The energy required to produce any perceptible effect at approximately $\lambda 3100\text{\AA}$ is more than 100 times as much as is necessary at $\lambda 2652\text{\AA}$. The energy values representing 100 per cent killing of the bacteria are far below the values having any measurable effect on the virus. For both of these biological materials, using the range of wave-lengths stated, the greatest influence is at $\lambda 2652\text{\AA}$. The resistance ratio of virus to bacteria, as represented by these results, is about 200:1. Relative to wave-length, graphs of certain "biological effects," collected from various authors, show striking resemblances.

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IRRADIATION OF PLANT VIRUSES AND OF MICRO-ORGANISMS WITH MONOCHROMATIC LIGHT

II. RESISTANCE TO ULTRAVIOLET RADIATION OF A PLANT VIRUS AS CONTRASTED WITH VEGETATIVE AND SPORE STAGES OF CERTAIN BACTERIA¹

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The study of the effect of monochromatic radiation on the virus of typical mosaic of tobacco and on *Serratia marcescens*, as described in the first paper of this series (Duggar and Hollaender, 1934), gave some striking results. It was found that the virus is extremely resistant to ultraviolet light at 0°C.; in fact, very much more so than *S. marcescens*. It was further found that the virus agent has its highest sensitivity at wave-length 2652Å.

This paper includes work on the resistance of *B. subtilis* (vegetative and spore form) and of the spores of *B. megatherium* f. to monochromatic ultraviolet radiation as compared with the resistance of *S. marcescens* and the virus of tobacco mosaic. To make possible a comparison of the results given in the first paper with those likely to accrue from this investigation, we used the same procedure except that the requirements of the species or stages of bacteria employed necessitated certain changes in the details of culture and suspension technique.

The preparation of a uniform suspension, that is, a suspension which will, upon further dilution, as in a series of poured plates, give the expected numerical ratios, often presents a difficult problem. With the organisms used in this study more time was necessary

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² This represents work done while holding a National Research Fellowship in the Biological Sciences.

in working out methods of overcoming these and other difficulties than in testing the organisms for their resistance to ultra-violet light. In fact, a number of organisms were investigated, but only a few of them were found suitable for our work. For each of these bacteria a special method of preparation was developed, including special media. However, care was taken that each suspension used by us checked in its light absorption fairly well with the standard suspension described in the earlier paper.

PREPARATION OF MATERIALS

Bacillus subtilis, vegetative stage. Stock cultures were prepared as potato-glucose agar slants, incubated at about 27°C. for forty-eight hours. Five standard loops of the surface culture were dispersed in 2 drops of a synthetic medium prepared according to Gottheil (1901).

KH ₂ PO ₄	0.5	gram
CaCO ₃	0.1	gram
MgSO ₄	0.25	gram
NaCl.....	0.1	gram
Ammonium tartrate.....	10.0	grams
Glycerin.....	10.0	grams
Cane sugar.....	5.0	grams
Distilled H ₂ O.....	1000	cc. + trace of iron

To 9 cc. of this medium in a test tube was added 1 cc. of the suspension; the culture was shaken thoroughly and filtered aseptically through sterile cotton into another sterile test tube. This liquid culture was then incubated for six and one-half hours at 28°C. The organism did not grow profusely in this time interval, nor did it form any pellicle or precipitate. After the incubation period mentioned, the material was again shaken and filtered through sterile cotton, the filtrate constituting the active bacterial suspension. The purified, pasteurized virus suspension (1:10), hereafter designated I. J. (infectious juice) was proximately purified by a diatomaceous earth treatment precisely as indicated in our previous paper. The experimental suspension employed was then prepared by adding 1 cc. of the active bacterial suspension and 11 cc. of the 1:10 virus suspension to 99 cc. of

physiological salt solution (hereafter designated S. S.), of one-half the strength previously used. This diluted the bacterial suspension approximately 100 times and afforded a virus suspension of 1:100 on the basis of the original I. J. Beginning with the preparation of the stock suspensions all materials were, as in the earlier work, plunged into an ice-water mixture, and so maintained up to the time the material was plated out.

To determine whether or not this *B. subtilis* suspension contained any spores, 5 cc. of this material were kept in a test tube in boiling water under constant shaking for five minutes and cooled at once. When cool, 1 cc. was removed and plated out in agar, and the freedom of the preparation from spores was shown by the lethal effect of this treatment, no colonies appearing, or, in exceptional cases, single colonies. A higher survival value would, of course, indicate the presence of spores.

Bacillus subtilis, *spore stage*. Stock cultures were prepared as for the vegetative stage, but these were incubated for ten days, the last three days being at 37°C. About 4 loops of this culture were then distributed in 2 drops of bouillon and pasteurized in boiling water for two minutes under constant shaking, thus killing the vegetative stage of the organism. Then 9 cc. of standard bouillon (8 grams bacto nutrient beef, in 1000 cc. water) were added, and the suspension shaken, and finally filtered aseptically through cotton.

As before, the final suspension contained 1 cc. of the bouillon culture + 99 cc. S. S. + 11 cc. I. J. 1:10, held in melting ice, as in the case of all prepared suspensions.

Bacillus megatherium sp., *spore stage*. The stock cultures, prepared as in the preceding case, were held four months (first four, weeks at 37°C.). Five loops of surface growth were added to 9 cc. S. S., shaken for five minutes, then pasteurized at 80°C. for ten minutes under constant shaking, and filtered through cotton. Of this suspension 1 cc. was used in preparing the exposure suspension as above.

Serratia marcescens, *vegetative stage (no spore stage occurring)*. Stock cultures were prepared on agar slants, as for the preceding, but incubation was for twenty-four hours only. Two loops of

this fresh surface growth were diffused in 9 cc. bouillon; then 1 cc. of this diffusion was added to a tube containing 9 cc. bouillon, and this last culture incubated at 28°C. for eight hours, during which interval no visible pigment develops. The exposure suspension, comparable to both of those preceding, was prepared with 1 cc. of this culture.

It should be noted again that the material consisting of 1 cc. bacterial suspension, 99 cc. S. S. and 11 cc. of virus suspension 1:10, was essentially optically clear, and had a very faint flavous tinge. The absorption of this material has been discussed adequately in our earlier paper. The number of bacteria varied between the limits 100,000 to 600,000; and a virus dilution of 1:100 gives under favorable conditions a disease incidence of 100 per cent.

In the case of all the organisms the controls indicate that the number of bacteria in the unexposed suspension remains relatively constant during the intervals of the several runs made.

APPARATUS AND PROCEDURE

In the first paper of this series there is given a description of the apparatus which makes possible the irradiation of biological suspensions with large and measured amounts of monochromatic light to $\lambda 2652\text{\AA}$. This apparatus was particularly serviceable for work centering upon the virus, relatively large amounts of energy being required. For the present study, however, with the work centering upon different forms of bacteria the requirements were somewhat different. Quantitative determinations of the effects of monochromatic light on bacteria require the handling of small and correctly measured quantities of radiation, for which a new apparatus has been constructed, fulfilling the conditions imposed.³ The monochromator used was not as light-strong as the instrument used in the earlier work, but (having

³ This apparatus has been built around a Bausch and Lomb quartz monochromator of the constant deviation type, loaned through the Radiation Committee, Division of Biology and Agriculture, National Research Council. A detailed description of this apparatus and of the method of irradiation of biological suspensions will be given in a separate paper. For this reason only a brief description of the experimental set-up will be here included.

crystalline quartz optics) it permitted work with radiation of shorter wave-length, especially with $\lambda 2537\text{\AA}$.

As a light source the capillary, quartz, mercury-vapor lamp was used, as in the earlier work, but in the construction of this lamp several changes were introduced (Hollaender and Stauffer, 1933).

The thermopile exposure-cell arrangement used in these studies is shown in figure 1. The arrangement is simpler than that

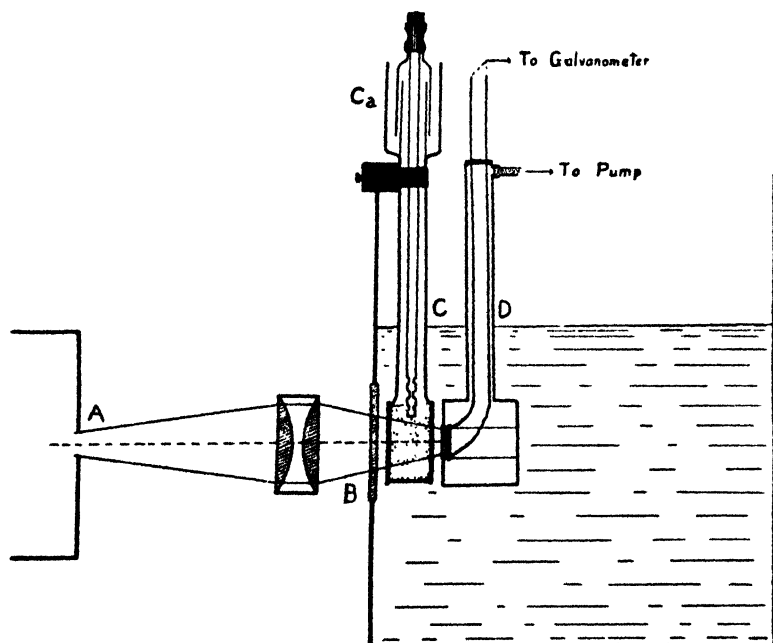


FIG. 1. THERMOPILE AND EXPOSURE-CELL ARRANGEMENT

A, exit slit of monochromator; B, quartz window of constant temperature tank; C, exposure-cell with stirrer (a, liquid seal); D, thermopile.

described in our previous paper, and it proved more efficient in the use of small amounts of energy. The thermopile was of the vacuum type, consisting of three bismuth-tellurium junctions, having a surface of 2 by 5 mm. It was connected to a type HS Leeds and Northrup galvanometer, and was read on a scale 2 meters from the galvanometer, 0.08 ergs/sec./mm. deflection, as standardized against a Bureau of Standards lamp. The thermo-

pile was constructed by Mr. J. P. Foerst of the Department of Physics. Since a thermopile of this degree of sensitivity is highly susceptible to vibrations, etc., special care was taken to prevent supporting any of the moving parts (stirrers, etc.) from the experimental work table, but rather directly from the ceiling or floor of the laboratory.

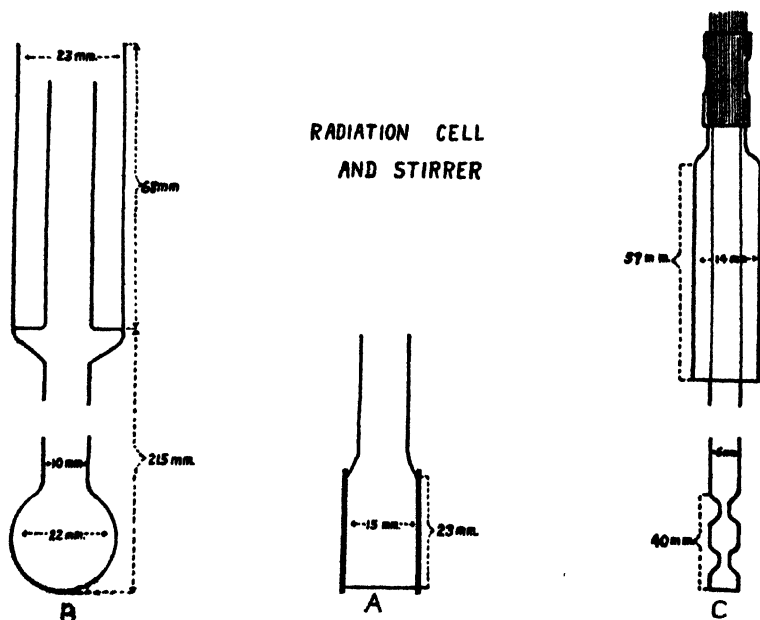


FIG. 2. EXPOSURE CELL

A, sectional view of cell proper; B, representing longitudinal section of entire cell; C, longitudinal section of stirrer and attachment.

A new type of cell was constructed which rendered possible the exposure of smaller amounts of biological materials, and this cell was likewise better suited for quantitative investigation, especially at shorter wave-lengths. It consisted of an inverted T tube with the horizontal tube cut close to the vertical on each side, the short cylinder thus resulting at the bottom being closed on each side with a crystalline quartz slip to serve as windows. This cell was equipped with a stirrer and a liquid seal (fig. 2).

It is perhaps important to emphasize one or two features in the operation of the stirrer. Exposure of the material was never started until the stirrer had rotated for at least three minutes. Moreover, since the stirrer made several hundred rotations per minute, all the material in the cell had abundant opportunity to come frequently into the path of the light, as referred to later. The extent of the area of the beam of light and the influence of stirring will, however, be considered more in detail in the proposed article on apparatus.

The following lines of the mercury spectrum in the ultraviolet were used: 2537, 2652, 2804, 2952, 3130, 3342, and 3652Å, some of these actually representing groups of lines not readily separated, as previously explained (Duggar and Hollaender, 1933).

The irradiation and the testing of the effects (i.e., the dilution plate series and the virus inoculation) were carried out as previously indicated. The arrangement of the exposure cell—the fact that not all the material was exposed to the radiation at every instant (fig. 1), but rather was brought into the beam of light at a rate of about 600 times per minute—brings up the question of the effect of short-interval interrupted exposures. This has been tested separately both with virus and with bacteria. The effects of the total energies in frequently interrupted exposures corresponded to those of the same energy at a single (continuous) exposure within the limits here considered. This special arrangement, whereby the irradiated material was practically surrounded by material not in the beam of light, simplifies the consideration of scattered light, the latter being largely reabsorbed so that it need not be treated separately.

DISCUSSION OF RESULTS

The present discussion, with an abundance of new data on other bacteria, may be regarded as supplementary to our previous account of the influence of monochromatic light on the virus of tobacco mosaic and on *S. marcescens*.

Our detailed results have been analysed and the salient features presented in smoothed graphs (figs. 3 to 7). The effect of the three longest wave-lengths (all in the near ultraviolet) have

been ignored in the graphs for the following reasons: Only very slight and not at all uniform results are recognizable at these wave-lengths. If only 1 per cent, or even somewhat less, of the large energies to which these organisms were exposed at those wave-lengths were of the very effective short wave-lengths, it would be more than sufficient in some cases to induce the effects obtained. However, we feel that this point merits a more care-

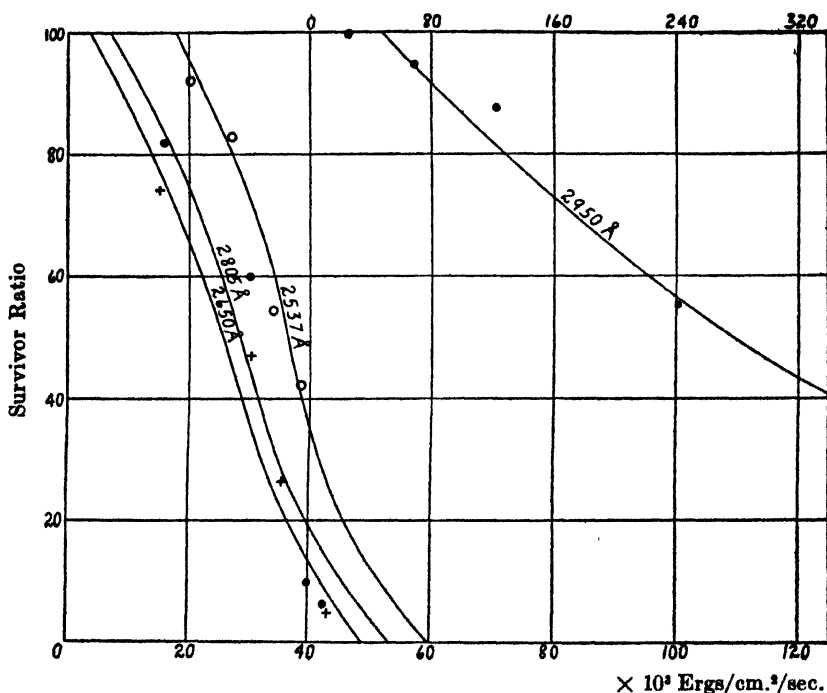


FIG. 3. *BACILLUS SUBTILIS*, VEGETATIVE STAGE ONLY (SEE TEXT FOR PREPARATION), SHOWING THE EFFECTS OF MONOCHROMATIC ULTRAVIOLET RADIATION BETWEEN WAVE-LENGTHS 2537 Å AND 2950 Å

ful investigation, which we hope we shall be able to make, at a later time, with a double monochromator.

Bacillus subtilis, vegetative stage. The relatively rapid rise of the curves for this organism (fig. 3) would indicate that a large number of bacteria are in an identical stage of sensitivity and development. Attention should be drawn to the different scale

used in representing the effects with $\lambda 2952\text{\AA}$, showing that the sensitivity of the organism decreases fairly rapidly toward $\lambda 3000\text{\AA}$. The maximum of sensitivity is at $\lambda 2652\text{\AA}$ (see also Gates, 1929). It is, furthermore, remarkable that this organism proves to be more sensitive to $\lambda 2805\text{\AA}$ than to $\lambda 2537\text{\AA}$. The absorption of the material is, however, higher at $\lambda 2537\text{\AA}$ than at $\lambda 2805\text{\AA}$. The larger part of this absorption is attributable to small amounts of

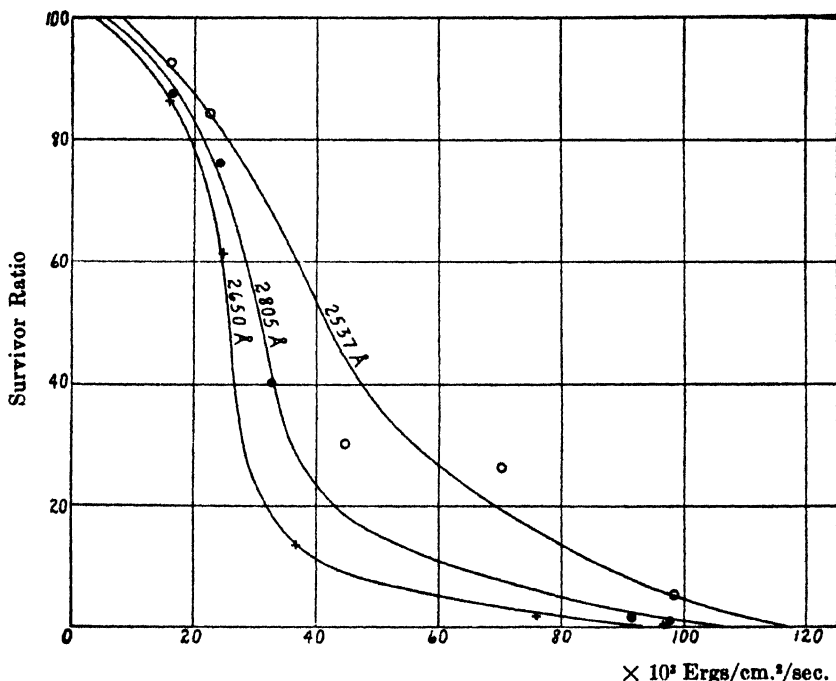


FIG. 4. *BACILLUS SUBTILIS*, SPORE STAGE (SEE TEXT-FOR PREPARATION), WITH GRAPHS REPRESENTING 3 WAVE-LENGTHS

other materials (flavones, soluble proteins, etc.) present in the virus suspension, and not to the bacteria present.

Bacillus subtilis, spore stage. The curves presented for this organism (fig. 4) have, in the higher survivor ratios in general, the same appearance as the curves for the vegetative stage. However, the exceedingly slow rise of the lower survivor ratios is remarkable. This would indicate a large variation in sensitivity

and probably the presence of a small number of more resistant spores. Previous investigators have found the spores to be about 2 to 7 times as resistant as the vegetative stage. Since they used methods which would not show the difference between high and low survivor ratios, their results may well be based, actually, on the presence of a few very resistant spores. The liquid sus-

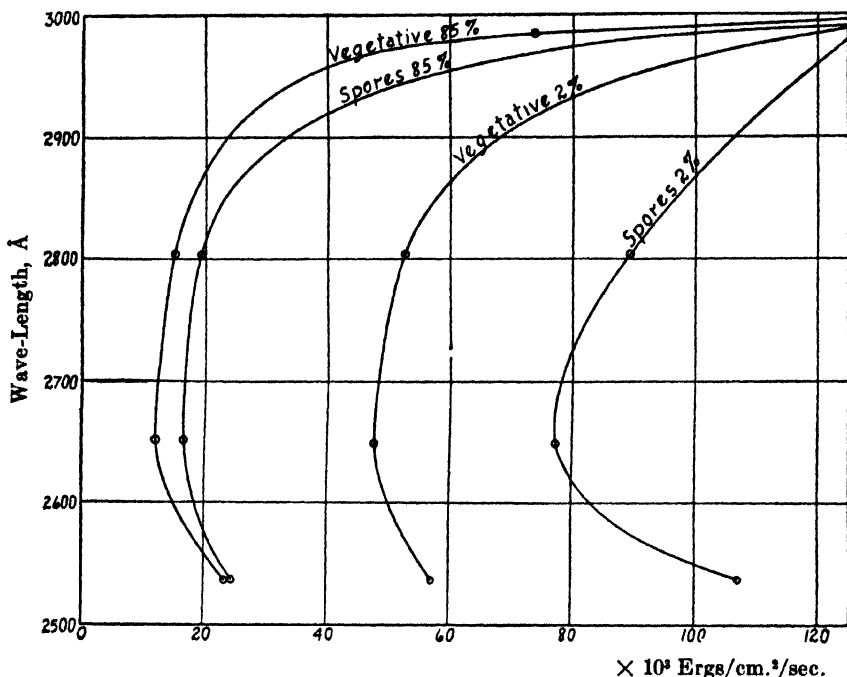


FIG. 5. *BACILLUS SUBTILIS*, SPORE AND VEGETATIVE STAGES AT 2 AND AT 85 PER CENT SURVIVOR RATIO, SHOWING, ESPECIALLY, GREATER DIFFERENCES, IN CONTRASTING THE STAGES, AT THE LOWER VALUES

pension and dilution count method avoids this error and gives precise information. Nevertheless, even with the suspension-dilution method, a very few organisms may survive through being held temporarily out of the beam of light, as for instance, in contact with the glass vessels, especially at the seams, but this is an extremely small factor by comparison with the survivor ratios in general. We feel that an attempt to get absolutely 100 per cent killing would give a very distorted picture.

It is apparent from figure 5 that the curves for the spore and the vegetative stage are generally conformable for any survivor ratio computed. More particularly, it is noteworthy that the 85 per cent survivor curves are close together, especially in the region of shorter wave-lengths; whereas the 8 per cent survivor curves are more widely separated. This difference may conceivably be related to different types of cell walls, or membranes, in the two stages. At $\lambda 2652\text{\AA}$ the ratio of the vegetative to the spore stage, considering a survivor value of 8 per cent, is approximately 4:5 in respect to energy level employed.

Apparently there is very little relation between heat resistance and light resistance. *B. subtilis* spores will stand boiling for fifteen minutes, the vegetative stage will scarcely withstand fifteen minutes at 65° . This point is brought out more emphatically in the graphs illustrating the behavior of the organism next discussed.

Bacillus megatherium sp., spore stage. By comparison with the data presented in the previous paragraph for the spores of *B. subtilis* it will be noticed that the level of energies required to produce similar effects with *B. megatherium* is distinctly higher (fig. 6). The large amounts of energy necessary may be related in this case also to the special characteristics of the cell walls. Otherwise, these curves show the same general features as those illustrating the relations of spores of *B. subtilis*. It is interesting, however, that *B. megatherium*, according to these data, exhibits a maximum sensitivity at $\lambda 2804\text{\AA}$, rather than at $\lambda 2652\text{\AA}$. The form of these curves, as plotted, is very suggestive of the type of curve indicating the effect of temperature on germination of spores of *Botrytis cinerea* (Henderson Smith, 1923) and other temperature effects. In this connection, attention may be drawn to the fact that the upper and lower stretches of these curves are based on fewer data and are, accordingly, not so exact as the mid-regions. Referring again to the relation between heat resistance and light resistance, it may be observed that the spores of *B. megatherium* will not withstand an exposure of more than ten minutes at $80^{\circ}\text{C}.$; yet these spores are more resistant to the effective rays than are those of *B. subtilis*, previously referred to, the heat resistance of which is considerably greater.

Resistance of the virus agent. In the previous paper the results obtained with the virus and with *S. marcescens* have been discussed. We shall now compare in one illustration (fig. 7) the virus with the two stages, so far as they occur, of the several species of bacteria thus far considered, the graphs being made on the basis of a survivor ratio of 50 per cent. That the virus would prove so much more resistant to radiation of these wave-lengths

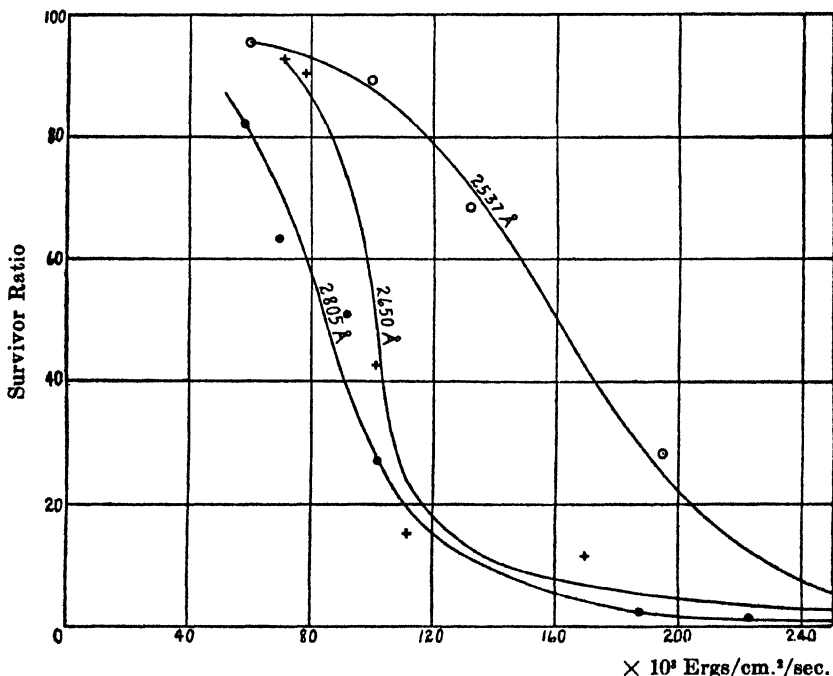
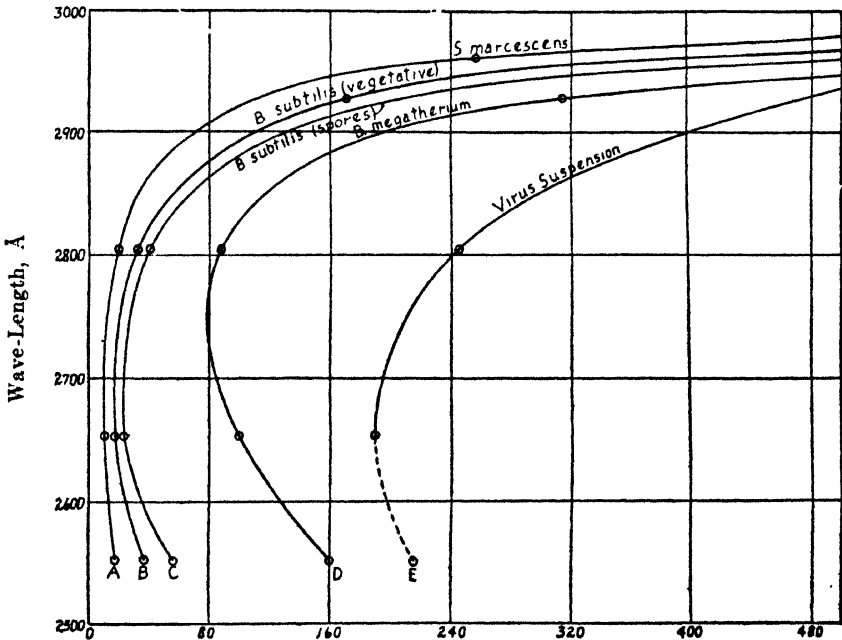


FIG. 6. *BACILLUS MEGATHERIUM*, SPORE STAGE ONLY

than spore stages of bacteria was not anticipated. However, these values again emphasize the lack of concordance with temperature effects. The virus is inactivated by an exposure of ten minutes at 90°C., and is therefore intermediate in heat resistance between *B. megatherium* and *B. subtilis*, spore stages.

It is well in this place to call attention again to the fact that the bacteria in the investigation reported here have been radiated in the virus suspension for the special purpose of securing

comparative data. For this reason it is not permissible to compare the absolute energies necessary to kill the bacteria in our case with the values obtained by other investigators. Practically all the quantitative work reported by others recently on bacteria has been done on an agar surface. Since we feel that the liquid suspension method has certain distinct advantages, we will at-



Curves A, B, C, D, $\times 10^5$ Ergs, curve E, $\times 10^6$ Ergs/cm.²/sec.

FIG. 7. RELATION BETWEEN BACTERIA (VEGETATIVE AND SPORE STAGES) AND VIRUS AT 50 PER CENT SURVIVOR RATIO, SHOWING CLOSE RESEMBLANCE IN SENSITIVITY RELATIVE TO WAVE-LENGTH, BUT ENTIRELY DIFFERENT ENERGY MAGNITUDES

tempt in another paper to approach a more precise quantitative investigation with this method, that is, one unaffected by the material necessarily added when the bacteria are used in comparison with virus.

While an absolute comparison of the results of our investigation with that of other investigators is not yet permissible, it is

interesting to compare the relative sensitivity to wave-lengths; further, to compare the sensitivity of vegetative and spore stages.

A very large number of papers have appeared on the sensitivity of bacteria to light. It is expected that a full review will appear in a forthcoming publication. However, several papers reporting work with approximately monochromatic light will be discussed.

In the work of Bayne-Jones and Van der Lingen (1923), *Staphylococcus aureus* was spread on an agar surface and irradiated behind a spectrograph. Lethal action began around $\lambda 3500\text{\AA}$, and continued with increasing effectiveness to the limits of transmission of the quartz instrument. Coblenz and Fulton (1924) irradiated *B. coli* spread on an agar surface with measured amounts of energy. Screens were used to separate fairly definite parts of the spectrum. The major part of the spectrum most effective in bactericidal action was found to be below $\lambda 3100\text{\AA}$. These investigators believed that they found a weak bactericidal effect up to $\lambda 3350\text{\AA}$. While there are slight bactericidal effects exhibited by our results up to $\lambda 3650\text{\AA}$, we feel that the small amounts of scattered light present may be responsible for these. In a series of investigations with *S. aureus* and *B. coli*, Gates (1929, 1929a, 1930) also employed the agar plate method, but used a monochromator. He found the maximum of sensitivity at $\lambda 2650\text{\AA}$.

Ehrismann and Noethling (1932) used a double monochromator and irradiated cultures on an agar surface with measured amounts of ultraviolet light. They found the highest sensitivity at $\lambda 2650\text{\AA}$ for *Micrococcus candidus*, *Bacillus pyocyaneus*, *Staphylococcus pyogenes-aureus*, *Saccharomyces cerevisiae* and *Vibrio Finkler*; at $\lambda 2805\text{\AA}$ for *B. prodigiosus*.

Pothoff (1921), working with *B. anthracis*, *B. subtilis*, and *B. mesentericus*, exposing these organisms in distilled water in water-cooled tubes, found the relation of the resistance of the spore and vegetative stages of the first organism as 6:1, the second organism 4:1, and the last 1.25:1. He likewise reviews the work done earlier in this field, so that consideration of such papers may here be omitted.

The results of certain other investigations have been contradictory, and mention need not be made of those in which the standard of comparison would be unsatisfactory.

SUMMARY

1. The present investigations have been made with a physical installation modified from that of our previous studies, and especially with a new type of exposure cell. A suspension technique and dilution culture procedure adjusted to the requirements of the organisms selected have been employed in a study of resistance of *B. subtilis* (vegetative and spore forms) and of *B. megatherium* (spore form) as compared with the resistance of *S. marcescens* and the virus of tobacco mosaic subjected to monochromatic ultraviolet radiation.

2. The results are given in the form of survivor curves for the different wave-lengths and for different intensities of the wave-lengths employed. The curves for spore and vegetative stages are generally conformable. The level of energies required to give a particular survivor value with spores of *B. megatherium* at any lethal wave-length used is higher than that required for a similar effect upon spores of *B. subtilis*.

3. While vegetative stages (*B. subtilis* and *S. marcescens*) are more sensitive to ultraviolet light, there is, in general, little relation between heat resistance and light resistance.

4. The resistance of the virus irradiated coincidentally and in the same suspension with the bacteria is so much greater than the resistance of spore stages as to be of a different order of magnitude.

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MORPHOLOGICAL RELATIONSHIPS OF SOIL MICROBES

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INTRODUCTION

The practical application of available knowledge in soil microbiology is a field still largely unexplored. With the exception of one group of bacteria, the symbiotic nitrogen-fixing bacteria, it is not yet possible to control the activities and functions of the soil flora or of specific groups of soil microbes in a practical manner in the field to the end that soil productivity may be maintained and improved.

While many species of soil bacteria have been isolated by the conventional use of gelatin or agar media, the method has proved to be wholly unsuitable for studies of the relative abundance and activity of the bacterial species in the soil, since these media favor a profuse development of the spore-formers and retard or even inhibit the growth of many other forms. Conn's (1918) direct microscopic technic, and Winogradsky's (1925) more recent methods for investigating species or groups of microbes, directly in the soil, or by means of highly selective solid media, have revolutionized old ideas regarding the predominance and importance of certain species or groups of soil microbes in their natural habitat. Direct microscopic soil examinations as first developed by Conn (1918) and since modified, have revealed a preponderance of spherical and coccoid forms and a relatively small number of the rod shaped spore-formers. The bacterial flora of the soil as Conn (1917) viewed it may be classified into two general groups, the spore-formers and the non-spore-formers. Apparently the spore-formers are not active under ordinary field conditions, and as expressed by Joffe and Conn (1923) they seize

upon fresh, available organic matter, preferably during periods of high moisture, so that their rôle in the soil under average conditions is one of *watchful waiting*. The non-spore-formers, consisting of small rods and cocci, make up 40 to 75 per cent of the soil flora, and according to Thatcher and Conn (1927) many of these organisms which develop as short rods on gelatin or agar plates always appear as small cocci when grown in soil. Thus it seems that the non-spore-formers as they exist in the soil consist largely of spherical and coccoid forms.

Winogradsky (1925), who used the direct microscopic method for studies of normal soils and of the same soils treated with various carbonaceous and nitrogenous organic substances, also found a preponderance of rounded or coccus forms and a relatively small number of the large rod-shaped forms in normal soils. He observed that the rod-shaped bacteria were highly stimulated by the presence of readily fermentable organic substances which caused intermittent, spasmodic activity, and he called them the *zymogenic* bacteria. To the members of the first group he ascribed the function of acting slowly but steadily on humic substances and called them the *autochthonous* flora, because of their indigenous nature.

There is much similarity between Conn's and Winogradsky's groupings in regard to both morphology and function. Morphologically the non-spore-forming and spore-forming groups of the former correspond respectively with the *autochthonous* and *zymogenic* groups of the latter. As to function, both investigators are generally agreed that the spherical and coccoid bacteria act slowly upon the humic substances in the soil, whereas the spore-forming, rod-shaped bacteria act vigorously, but spasmodically on the readily fermentable substances, which under field conditions consist largely of the crop residues and farm manures that are returned to the soil.

If it is possible, by means of the direct method, to classify the bacterial flora of the soil into two morphological groups with their corresponding functions, then additions of fresh, easily fermentable organic substances or repeated applications of organic residues to the soil might well be expected to cause significant

fluctuations or more or less permanent changes in numbers of the two groups. These fluctuations or changes, if they actually occur under field conditions, could be detected by periodical microscopic examinations of the soil and the direct method would then serve a useful purpose, in determining not only the relative activity of these two groups of organisms in soils containing different amounts of organic matter, but also the relative importance of their functions in the progressive steps of soil organic matter decomposition. In an attempt to gain more definite information regarding these possible relationships, the relative numbers in the *autochthonous* and *zymogenic* groups were determined at short intervals in two soils of the same type to which had been added different amounts of organic residues in the field over a long period of time and which were further subjected to various treatments in the laboratory.

EXPERIMENTAL PROCEDURE

Samples of Palouse silt loam, a fertile prairie soil, were obtained from two of a series of field plots used for experimental work during the previous twenty-seven years. One of these plots had received an annual fall application of manure at the rate of 12 tons per acre and had grown a crop of winter wheat every year. The other plot had not received any fertilizer and had been in winter wheat and summer-fallow in alternate years. The soil samples for investigation were taken after a crop of wheat had been harvested from both plots. Summer precipitation being scarce in this area, the soil in fields cropped with cereals is usually dried out during July, August, and also in September until the fall rains come. As normal microbial activity cannot take place in dry soil, the samples, consisting of surface soil 8 inches in depth from several places on each plot, were not obtained until about ten days after the first fall rains, which occurred in October, had thoroughly moistened the soil to a depth of 10 inches. The moist soils were spread out evenly and allowed to dry at room temperature until they could be handled without danger of puddling. They were then passed twice through a 4-mesh screen to make sure of thorough mixing and of the removal

of stubble and coarse plant roots. A portion equivalent to 3 kgm. of dry soil was weighed out from each sample and water was added until the moisture content and consistency of the soils were similar to those occurring in the field when it is in the best condition for tillage. The moisture content of the manured soil in this condition was 21 per cent and that of the other soil 19.5 per cent. Each portion was divided into two equal amounts equivalent to 1500 grams of dry soil and these were placed in $\frac{1}{2}$ -gallon, wide-mouthed bottles. The bottles were connected with an absorption train for carbon dioxide determinations for the purpose of estimating the total microbial activity in the soils.

During the course of the experiment the moisture content of the soil in the bottles was maintained within narrow limits by additions of water when necessary. The work was carried on under average room temperature, which ranged from 20° to 25°C.

Microscopic examinations of soil samples were made every two days during the first three weeks, and every three or four days thereafter. A butter sampler was used to procure two cores from the whole depth of the soil in each bottle at each sampling. These cores were thoroughly mixed and 1 gram of the mixture was used for microscopic examination. The remaining part of the sample was returned to the bottles which were thoroughly shaken to fill the holes made by the sampler. The carbon dioxide collected in the absorption train was determined each time the soils were sampled.

Following the first three months of observation, the soils were removed from the bottles and treated with calcium carbonate. In addition, finely ground filter paper mixed with sufficient sodium nitrate to make the nitrogen content of the filter paper equivalent to 2.5 per cent was added to one of each two duplicate portions of soil. The arrangement of the soils and the treatments are shown in table 1.

The ingredients were thoroughly mixed with the soils, which were returned to the bottles. Samplings were made in the same manner as described for the first period of observation. Since the most intensive microbial activity was expected to take place

during the first days following the addition of the organic material, the soils were sampled daily during the first eight days, after which the time interval was gradually increased until it was seven days between samplings beginning with the sixth week.

Conn's (1929) technic with slight modifications was adopted for the microscopic examination of the soils. Instead of using 0.015 per cent gelatin in making the soil suspensions as recommended by Conn, 0.1 per cent agar was substituted, and erythrosin (extra) was used for staining fluid instead of rose bengal.

Two smears were prepared from each sampling and five microscopic fields were counted on each smear. Thus the number of

TABLE 1

NUMBER OF SAMPLE	PLOT TREATMENT	ADDITIONAL SOIL TREATMENT	
		<i>per cent</i>	
1	{ Not manured; winter wheat alternate years	CaCO ₃	1
2		CaCO ₃	1
		Filter paper	1
3	Manured—12 tons per acre for 27 years; winter wheat every year	CaCO ₃	1
4	{ Manured—12 tons per acre for 27 years; winter wheat every year	CaCO ₃	1
		Filter paper	1

organisms recorded represents the average count of ten fields. In making counts, only those organisms that could be definitely recognized as coccoid or rod-shaped forms were considered and all doubtful forms were ignored. Therefore, no pretense is made that the numbers reported represent the actual numbers occurring in the soil. It is thought, however, that the method can be used for making dependable observations regarding changes in numbers and in numerical relationships between the two morphological groups studied.

EXPERIMENTAL RESULTS

Duplicate portions, as nearly identical in all respects as it is possible to make them, were prepared for each of the two soils to

TABLE 2

Microorganisms in millions per gram of dry Palouse silt loam

DAY	SAMPLE 1 NO MANURE		SAMPLE 2 NO MANURE		SAMPLE 3 MANURE 27 YEARS		SAMPLE 4 MANURE 27 YEARS	
	Microbial forms							
	Coccoid	Rod- shaped	Coccoid	Rod- shaped	Coccoid	Rod- shaped	Coccoid	Rod- shaped
1	17.22	22.43	21.59	17.22	31.80	37.73	26.03	28.55
3	16.60	24.60	12.57	20.50	28.40	31.75	26.90	32.42
5	11.92	21.50	13.25	29.15	26.87	34.45	29.18	38.42
7	22.55	26.65	16.40	20.50	32.80	32.80	32.80	38.95
9	20.50	30.75	16.65	22.80	32.80	35.83	36.90	37.10
11	30.75	30.75	20.75	20.50	28.70	30.75	28.70	38.95
13	22.48	27.80	15.21	32.56	38.95	24.60	30.75	30.75
15	11.92	19.91	15.21	22.56	32.43	20.51	27.82	27.13
17	25.86	18.01	18.55	15.90	36.90	32.80	30.75	26.50
19	28.70	20.50	24.60	20.50	24.57	18.25	25.17	17.86
22	22.52	22.52	19.87	17.22	21.83	23.80	26.50	24.48
25	17.22	17.86	15.90	22.52	30.75	28.70	28.70	22.50
28	24.60	26.65	16.40	22.55	28.75	26.65	36.90	30.75
31	22.55	20.50	18.45	16.40	28.70	24.60	33.78	30.09
34	18.55	17.12	15.90	17.86	20.56	21.62	25.81	35.05
38	19.35	20.74	20.50	22.55	36.90	27.70	31.67	35.83
41	16.55	17.83	23.25	15.90	22.55	28.46	27.82	25.17
44	17.38	20.50	15.41	18.70	30.75	28.27	29.18	24.60
48	18.45	22.55	20.50	12.30	27.53	24.60	29.68	22.96
52	15.25	21.30	12.57	20.35	37.10	26.50	25.51	31.71
55	16.55	20.50	17.83	22.50	28.27	22.55	30.75	24.60
58	18.55	26.65	15.25	20.35	32.43	30.75	35.85	26.65
61	17.35	23.53	16.40	22.55	28.70	26.65	28.70	36.90
65	22.00	18.70	26.65	21.48	36.90	28.70	38.95	24.60
69	20.50	28.70	24.60	24.60	34.85	26.65	36.90	26.65
73	22.55	24.60	22.55	26.60	32.80	28.27	36.90	32.80
77	22.00	30.80	24.60	26.65	28.70	38.95	32.80	24.60
81	30.00	29.75	22.55	26.65	32.80	30.75	34.85	28.70
85	20.50	24.45	18.45	24.60	32.85	28.27	30.75	35.95
89	16.40	26.65	22.55	22.55	34.85	30.75	28.70	34.85
93	16.40	26.00	18.45	26.65	38.95	26.95	32.50	28.50
Average. . .	20.12	23.57	18.82	21.73	31.02	28.37	30.91	29.85

determine if the numbers of organisms in the two morphological groups under consideration, or the total microbial activity, would differ materially between duplicate samples during a three-month period of observation. The results of the microscopic

examinations are given in table 2, and the results of the carbon dioxide determinations are shown graphically in figure 1.

The results given in table 2 indicate that on an average the numbers of both the coccoid and rod-shaped organisms were slightly greater in sample 2 than in its duplicate, sample 1. In the manured soil the number of coccoid forms was slightly greater in sample 3 than in its duplicate, sample 4, but the reverse was true of the rod-shaped forms. The total microbial activity in duplicate samples was surprisingly similar, as may be noted in

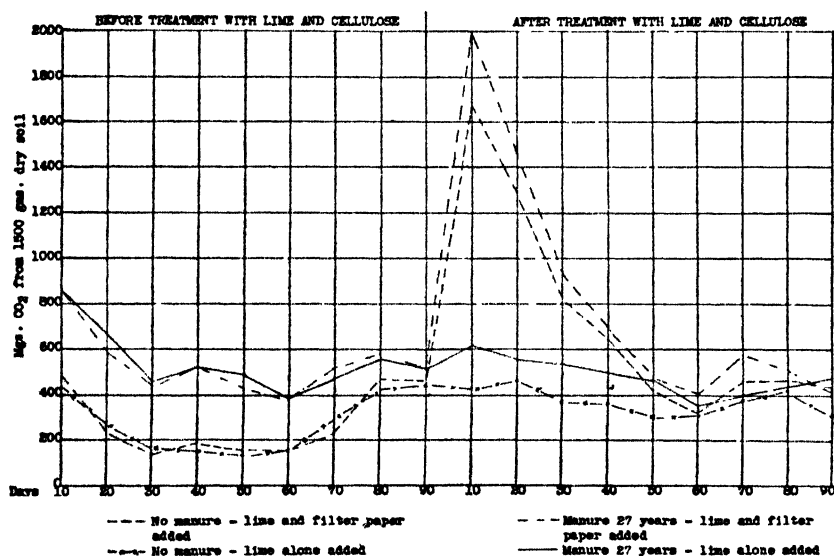


FIG. 1. EVOLUTION OF CO₂ FROM PALOUSE SILT LOAM IN TEN-DAY PERIODS

figure 1 which shows the amounts of carbon dioxide liberated at intervals of ten days. Taking into account the limitations of the microscopic method, even though it is perhaps the most accurate method available, and the fact that the amounts of carbon dioxide liberated in duplicate samples were so nearly alike, it is safe to assume that in general the microbial activities in duplicate samples were similar and that the relatively small differences in numbers of organisms observed are probably not significant.

Judging from the numbers of organisms and the amounts of carbon dioxide recorded, the microbial activity in the soil that had not been manured was markedly less than in the manured soil. On an average the number of coccoid forms in the untreated soils was about two-thirds of that in the manured soil and the number of rod-shaped forms in the former was approximately three-fourths of that in the latter. In the non-manured soil the rod-shaped forms were generally more numerous than the coccoid forms, whereas this tendency was reversed in the manured soil, especially following the first ten days of observation. The fluctuation in numbers in both groups was relatively small throughout the three-month period of observation, and at no time were any of the observed tendencies to apparent changes in numerical relationships between the two groups definite enough to be significant. Obviously the repeated application of manure to the soil over an extended period of time has resulted in a marked increase in numbers in both the autochthonous and zymogenic groups and to some extent in the numerical relationship between those groups. As both of these changes seemed to be more or less of a permanent nature, they might be considered significant if it were not for the fact that the differences in the amounts of carbon dioxide evolved from the two soils appear to be out of proportion with the observed differences in numbers of organisms. The quantities of carbon dioxide liberated, as shown in figure 1, were about twice as great in the manured as in the non-manured soil, whereas the corresponding difference in numbers of organisms was much less. Assuming that the evolution of carbon dioxide is a general index of the total microbial activity in the soil, it is clear that the apparent significance of the observed differences in the autochthonous and zymogenic groups cannot be properly evaluated unless the activities of the other microbes that are invariably present in the soil are in some way accounted for. Since it is generally accepted that the addition of calcium carbonate and of organic substances to the soil brings about marked changes in microbial activities, the treatments outlined in the experimental procedure for the four samples under consideration should yield data that are indirectly helpful in

partially accounting for the activities of the soil microbes that do not belong to the two groups studied. Furthermore, these same soil treatments, since they include applications of carbonaceous material in the presence of liberal amounts of readily available nitrogen, should also produce additional information concerning the relative importance of the two morphological groups in the progressive steps of organic matter decomposition. The results of the microscopic examinations and carbon dioxide determinations made following these treatments are recorded in table 3 and figure 1 respectively.

It is noted from the data in table 3 that the addition of 1 per cent of calcium carbonate alone stimulated the development of both the coccoid and rod-shaped organisms in the non-manured soil, whereas in the manured soil it seemed to result in some increase in the number of rod-shaped forms during the first two weeks, but caused no marked change in the number of coccoid forms. Thus, the addition of lime resulted in somewhat larger average numbers of the combined morphological forms, but the total microbial activity as indicated by the carbon dioxide evolution, which is recorded in figure 1, at intervals of ten days, remained approximately the same.

When filter paper was used in addition to the lime, however, there was a sudden marked increase in the number of rod-shaped forms in both soils. This change took place spontaneously, starting from the first day and continuing for about a week. It was much more pronounced in the non-manured than in the manured soil. The coccoid forms did not seem to be affected by this treatment, as there was no marked change in numbers in either soil. Thus, the evidence is strongly in support of the contention that the coccoid group acts predominantly but slowly upon the humic substances, whereas the rod-shaped forms act vigorously upon the readily decomposable carbonaceous substances in the presence of available nitrogen. These results are in general agreement with those obtained by Joffe and Conn (1923) for their spore-formers, and by Winogradsky (1925) for his zymogenic bacteria, when large quantities of various carbonaceous substances containing readily available nitrogen were

TABLE 3

Microorganisms in millions per gram of dry Palouse silt loam treated with lime and with or without one per cent filler paper

DAY	SAMPLE 1 NO MANURE LIME + FILTER PAPER		SAMPLE 2 NO MANURE LIME		SAMPLE 3 MANURE 27 YEARS LIME + FILTER PAPER		SAMPLE 4 MANURE 27 YEARS LIME	
	Microbial forms							
	Coccoid	Rod- shaped	Coccoid	Rod- shaped	Coccoid	Rod- shaped	Coccoid	Rod- shaped
1	22.55	61.43	20.50	32.80	32.80	45.43	30.90	42.60
2	24.60	49.20	28.10	36.90	28.70	43.05	28.70	40.05
3	24.10	48.50	28.10	36.95	28.10	49.20	24.60	41.50
4	28.70	61.50	24.60	34.85	26.65	46.55	28.00	45.10
5	26.65	38.95	26.65	45.10	30.80	48.50	22.60	40.10
6	30.75	36.90	26.65	34.85	34.85	45.10	32.80	42.60
7	26.65	43.05	28.07	38.90	30.70	36.90	28.70	36.90
8	26.65	32.80	24.50	38.95	28.07	38.95	30.70	36.90
10	24.60	38.95	22.55	32.80	28.70	36.90	22.55	40.10
12	26.65	32.50	28.70	30.75	24.50	38.80	26.65	32.80
14	22.55	38.25	26.65	34.85	30.70	45.10	28.10	34.85
17	28.50	36.90	22.50	28.70	32.80	42.60	30.80	38.95
20	22.55	43.05	18.10	30.75	28.70	53.30	20.50	30.75
24	32.80	46.05	16.45	36.90	26.65	41.00	22.55	30.75
27	30.01	35.50	26.65	32.80	20.75	49.20	28.10	34.20
31	32.85	38.70	28.70	32.80	28.75	36.90	32.80	36.90
34	24.60	41.00	22.55	34.85	34.80	45.10	26.65	34.00
38	28.70	36.90	30.75	34.85	32.80	38.95	30.75	41.00
45	26.55	41.00	24.60	26.65	30.80	38.95	28.70	42.15
52	26.65	32.80	28.70	32.80	32.80	36.90	30.90	34.85
66	28.70	32.80	24.60	30.75	32.80	34.85	26.65	34.85
73	24.60	36.90	26.65	28.70	36.90	38.95	26.65	34.85
80	24.60	26.65	22.65	26.55	30.75	41.00	24.60	32.80
87	23.50	28.16	22.55	28.70	32.80	38.95	26.65	30.99
94	22.35	29.68	22.80	30.75	31.82	37.99	27.76	33.94
101	24.60	29.50	26.65	27.75	28.92	30.99	22.72	30.99
108	22.35	22.35	22.35	30.99	33.05	37.18	24.79	35.12
115	20.66	20.66	18.95	22.75	26.75	33.05	20.65	33.05
Average...	26.03	37.88	24.68	32.70	30.61	41.08	27.01	36.57

applied to the soil. It should be mentioned in this connection that the observed numbers of rod-shaped organisms, which are here taken to represent the spore-formers, and zymogenic bacteria of the forementioned authors, are probably larger than they

estimated in their investigations. This should not be surprising in view of the fact that in this work the actinomyces appearing as rod-shaped conidia were probably included with the rod-shaped organisms. As to the coccoid organisms, it is quite probable that the numbers here reported are too low, but this can be explained by the fact that only those morphological forms which could be definitely recognized as coccoid or rod-shaped organisms were included in the counts. Since many of the soil bacteria are extremely small, by far the larger part of those that were rejected would probably fall in the coccoid group. These discrepancies, however, appeared to be of minor consequence in the final results.

Although the addition of lime and of filter paper to the soils caused distinct increases in numbers as well as definite changes in numerical relationships between the two morphological groups of organisms under consideration, these changes were by no means commensurate with the enormous stimulation in the total microbial activity as represented by the amounts of carbon dioxide liberated at intervals of ten days. This is graphically illustrated in figure 1. The increase in total microbial activity as indicated by carbon dioxide production was out of proportion with the comparatively small increase in numbers of autochthonous and zymogenic organisms not only in terms of quantity, but also in terms of time, since the former lasted over a period of four to five weeks, while the latter subsided after the first week. Evidently other organisms, probably largely filamentous forms, took an important part in the biological activities in these soils, and their part was not accounted for by means of the microscopic technic used, nor could it have been accounted for by this means in view of the fact that the relatively infrequent occurrence of filamentous forms appearing on the prepared smears seemed wholly inadequate for that purpose.

The data obtained in this work are convincing evidence that rather permanent changes in numbers of autochthonous and zymogenic organisms and in numerical relationships between the two groups resulted from repeated applications of organic residues to the soil. They also show that large applications of lime and

filter paper to the soil effected marked increases in numbers as well as a definite change in numerical relationships between the two groups, thus indicating in some degree the respective functions of the autochthonous and zymogenic groups in the progressive stages of decomposition of organic matter. However, the observed changes in microbial numbers and relationships were not commensurate with the total biological activity as measured by the evolution of carbon dioxide from the soil. Therefore, the microscopic method, if used to study the functions of the soil microflora through the activity of the two morphological groups here under observation must be considered inadequate as a means for accurate studies of the functions of the entire soil microflora in its natural habitat, especially of those functions pertaining to processes that are directly or indirectly concerned with the decomposition of organic matter. The reason is that the method does not properly account for the activity of the filamentous organisms or for certain important functions of specific physiological groups of bacteria. When used as a supplement to the plate method in which highly selective media for specific physiological groups of organisms are employed, the microscopic method should be well adapted for quantitative studies of bacteria and for qualitative determinations of changes in the numbers and activity of the predominating soil flora.

SUMMARY

Periodical microscopic examinations were made to determine the number and numerical relationships of the coccoid (autochthonous) and rod-shaped (zymogenic) organisms in two soils that had received different amounts of organic residues in the field for many years and were further subjected to various treatments in the laboratory.

Repeated applications of manure over a period of twenty-seven years has resulted in a marked increase in numbers in both the autochthonous and zymogenic organisms in the soil and to some extent in the numerical relationships between these groups.

The application of 1 per cent calcium carbonate to the soils resulted in larger average numbers of organisms in the two mor-

phological groups combined, without causing any marked changes in the amounts of carbon dioxide evolved.

The addition of filter paper in the presence of readily available nitrogen caused a large increase in numbers of rod-shaped organisms in both soils during the first week. The activity of the coccoid forms did not seem to be affected by this treatment.

Although a definite increase in numbers of organisms and a distinct change in numerical relationships between the two morphological groups resulted from the addition of organic substances, these changes were not commensurate with the increase in total microbial activities as measured by the carbon dioxide liberated from the soils.

The microscopic method used as a means of determining the activities of the two morphological groups under observation is inadequate for accurate studies of the functions of the soil microflora, but should be well adapted for quantitative studies of bacteria and qualitative changes in numbers of the predominating soil flora if used in connection with the plate method.

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STUDIES ON PNEUMOCOCCUS VARIATION

I. VARIANTS CHARACTERIZED BY RAPID LYSIS AND ABSENCE OF NORMAL GROWTH UNDER THE ROUTINE METHOD OF CULTIVATION

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INTRODUCTION

The pneumococcus variants to be described in this paper were first observed during a series of studies on the smooth to rough transformation. These new variants are characterized by a tendency to undergo a rapid spontaneous lysis under certain cultural conditions at 37°, and some of them grow much more slowly than do the ordinary strains of pneumococcus. This characteristic behavior is best detected by growing these variants on blood agar at 37°. Under these conditions the effects of the lytic process and speed of growth on the colony form are most evident. With some strains the lytic process commences a few hours after cell division has started, the result being that the pneumococcus cells are completely lysed and apparently non-viable before the colonies attain a size visible under the colony microscope, and there is no appreciable growth at twenty-four hours. In other strains the lytic process begins later so that colonies varying in size from 0.1 mm. to 1 mm. are produced during a growth period of between eight and twenty-four hours, and these colonies subsequently undergo a rapid lysis thus acquiring a transparent phantom-like appearance at the end of twenty-four hours.

For the sake of brevity the letters P-C will be used to designate all of the pneumococcus variants which show this tendency to lysis and consequently form small phantom colonies or fail to grow appreciably on blood agar at 37°.

The P-C variants not only have been produced by artificial methods in the laboratory but have also been isolated directly from cases of pneumococcus infection in man. They appear to result from a dissociative process other than that which produces the ordinary rough to smooth transformation, both virulent and avirulent variants exhibiting lytic properties. However, there are probably important connections between these two dissociative processes. The resemblance of the colonies of the P-C variants, under certain conditions, to G colonies of Hadley (1931) is rather striking. This aspect of dissociative phenomena will be discussed at the end of this paper.

Phantom colony variants of the anthrax bacillus which produced numerous atypical forms after twenty-four hours incubation were described by Nungester (1929). It seems likely that certain of the lytic variants of the Shiga dysentery bacillus described by Twort (1920), Arkwright (1921) and others were not cultures containing a bacteriophage but were closely similar to the type of variant to be described in this paper. Hadley (1927) mentions certain lytic variants which are apparently not due to the direct action of the bacteriophage to which he applies the term suicide cultures. Numerous other examples of what appears to be exaggerated autolysis may be found in the literature.

Dawson (1928) has reported small and lytic colony forms of the pneumococcus among what he considered to be intermediate variants. More recently this same author (Dawson, 1933) has reported a new type of rough variant of the pneumococcus which exhibits extreme pleomorphism. Rakieta (1930) obtained from a virulent type II culture a variant which grew better at 20°C. than at 37°C., and also a variant showing very atypical morphology and forming microscopic colonies. Blake and Trask (1933) also report a small colony form intermediate between rough and smooth.

The present work is concerned with methods of producing the P-C variants, with the effect of the characteristic lytic process on their colony form and cell morphology, and with the effects of simple chemical and physical changes such as acidity, oxygen

tension, carbon dioxide tension, and temperature in hastening or preventing the lytic process. The latter part of the paper is concerned with methods of causing the P-C variants to revert to normal strains, and with studies on the antigenic composition and virulence of the P-C variants.

The effect of temperature on the lytic process must be mentioned at this time because it has an important bearing on the methods of isolation, cultivation, and artificial production of the P-C variants. Lowering the temperature inhibits the lysis of the P-C variants so that although they grow scantily or not at all at 37° these P-C variants give a type of growth indistinguishable from that of normal strains when cultivated on blood agar at 25°C. for three days. This observation made it possible, simply by cultivation at 25°, to carry the variants in a relatively stable form in stock culture, to isolate phantom colony forms from cases of human infection, and to devise an effective method for the artificial production of P-C variants.

METHODS

The media used in the study of the P-C variants were as follows:

- (1) Five per cent horse blood infusion agar.
- (2) One per cent glucose infusion agar.
- (3) Infusion broth containing one drop of rabbit whole blood to each 5 cc.
- (4) One per cent glucose infusion broth.

In cultivating P-C variants and studying the effects of temperature, two incubators, one maintaining a temperature of 25°C., and one a temperature of 37°C., were used. In all cases unless otherwise specifically noted the pH of the media was 7.6 and the conditions of O₂ and CO₂ tensions those of the laboratory air.

The process of lysis and the accompanying changes in morphology in the P-C variants were observed by growing the organisms on blood or serum agar on a cover slip inverted over a hollow ground microscope slide, and watching the development of the colonies under the microscope.

Studies of the effect of oxygen and carbon dioxide tensions

were conducted by placing the cultures in closed jars and pumping out the air to the desired tension, then replacing with nitrogen, or carbon dioxide, or both. The pH was varied by adding lactic acid or sodium hydroxide to the media and checking on a colorimetric scale.

The cultures used in the study of antigenic properties and virulence were grown in rabbit-blood broth at 37°, or on blood agar at 25° and then emulsified in broth. Autolysates were made by adding 0.5 per cent of phenol to the forty-eight hour blood broth cultures and allowing them to stand for three days. The autolysates were then boiled successively at pH 8.5 and 4.0 to remove antigenic constituents which might interfere with the precipitin titration for specific soluble substance. The pH was then brought back to 7.0, the precipitated protein centrifuged off, and the supernatant used for the precipitin tests.

PRODUCTION OF P-C VARIANTS BY DISSOCIATION OF STOCK CULTURES AT 37° AND 25°

P-C variants of the pneumococcus exhibiting the characteristic lytic phenomenon were discovered during attempts to convert an unstable type I, rough strain into a smooth by growth in a special potato extract medium. After incubation for twenty-four hours in the potato medium, the R culture gave, on plating to blood agar, a mixture of colony types.

Selection and subculture of some of the colonies yielded a strain which showed distinct lysis in twelve hours on blood agar at 37°. This strain was moderately virulent for mice, being recovered unchanged from the heart blood.

Similar P-C variants were obtained using the following methods to produce the dissociation.

1. From type I S and R strains by growth in human whole blood for ten to twenty generations at 37°.
2. From Type I S by growth in 10 per cent type I antiserum broth for five generations at 37°.
3. From type I S by growth on an agar medium containing 1 per cent peptone, 1 per cent laked horse red corpuscles, and 0.5 per cent glucose at 37° for three generations.

4. From the type I S stock culture by a dissociation of unknown cause which occurred during the routine procedure used to maintain virulence, e.g.: daily transplants in rabbit-blood broth and mouse passage once a week. The P-C variants later disappeared from the stock culture.

5. From types I, II and several strains of group IV by the daughter colony dissociation which occurs during cultivation on blood agar at 25°.

In all cases the P-C variants were isolated by subcultures on 5 per cent horse-blood agar and colony selection.

The fifth method mentioned above, namely, the daughter colony dissociation at 25° was found to be the most satisfactory method for the artificial production of P-C variants from smooth cultures. The culture is plated on blood agar so that well separated colonies are formed, and is then allowed to grow at 25° for three to eight days. At varying times during this period raised patches or papillae appear on the flattened colonies. The appearance of these papillae indicates the beginning of dissociation and if the colony is subcultured at this time or later a few dissociant colony forms are obtained, all or part of which may be P-C variants. Farago (1932), describes a similar dissociation of the pneumococcus at 37° but this author did not succeed in isolating any dissociants.

MORPHOLOGY OF THE COLONIES OF P-C VARIANTS WHEN CULTIVATED ON BLOOD AGAR AT 25°C.

1. Colony characteristics of type I

As indicated in the introduction, most of the P-C variants will produce colonies 1.0 mm. to 3.0 mm. in diameter which are similar to those of normal smooth strains when grown on the blood agar at 25° for three to five days. When cultures which have formed daughter colonies are subcultured on blood agar at 25° several dissociant colony types are obtained. Among these the three that occur with greatest frequency are as follows: (1) Large, smooth, shining, convex opaque; (2) large, smooth, dull, flat, translucent; and (3) small, smooth, slow growing, shining convex.

Of these three colony forms at 25° one, two, or all three may exhibit the lytic properties characteristic of P-C variants when cultivated at 37°. Studies on the dissociation of several strains of type I obtained from different sources showed that the lytic properties are not all equally marked in dissociants from different strains.

The production of dissociants giving the translucent and opaque colony types at 25° takes place with great regularity in the daughter colony dissociation of practically all pneumococcus cultures. Similar translucent and opaque colony forms have been observed in normal smooth strains when grown at 37°. In all cases these properties are permanent and probably represent a third type of variation in addition to the P-C variation and the S to R variation.

For the purposes of the present discussion no important differences exist between the P-C variants which give convex opaque colonies at 25° and those which give flat translucent colonies at 25°. Both forms show all of the typical P-C characteristics. The fact that the appearance of the lytic properties characterizing the P-C variation coincides with the appearance of the opaque and translucent variants undoubtedly has some, as yet undetermined, significance. Further work on the translucent and opaque colony forms is now in progress.

The small colony P-C variants (third colony form listed above) which are obtained less regularly than the larger colony forms seem to differ most from the normal smooth form. They show marked departure from the latter in cultural characteristics, grow slowly, and are usually of low virulence. When kept on blood agar at room temperature they are very stable.

2. Colony characteristics of types II, III and IV P-C variants

The P-C variants obtained from type II by daughter colony dissociation at 25° are in general very similar to those obtained from type I except that slow-growing small-colony variants occur relatively more frequently in the type II dissociant cultures. Variants corresponding closely to those described by Rakieten¹ (1930) were obtained in some cases.

¹ See Introduction.

No P-C variants could be obtained from the stock type III cultures. However, a typical P-C strain of type III giving no growth or phantom colonies on blood agar at 37° and normal smooth transparent colonies at 25° has been isolated from the sputum of a case of type III lobar pneumonia.

DIRECT ISOLATION OF PNEUMOCOCCUS P-C STRAINS FROM CASES OF
HUMAN INFECTION BY CULTIVATION AT 25°

It has just been indicated that P-C variants of the pneumococcus have been obtained not only from stock laboratory cultures but also directly from material derived from pneumococcus infection in man. Samples of sputum from cases of lobar pneumonia were washed and plated directly on blood agar, and incubated at 25°. After two or three days colonies that appeared to be pneumococcus were transferred to fresh blood-agar and one set of these was kept at 25°, the other at 37°. Blood cultures were taken in the usual way in flasks containing 100 cc. of broth and incubated for twenty-four hours at 37°. The culture was then plated on blood agar at 25° and 37°. The usual methods of identifying the organisms were used and all were tested for virulence by mouse injection.

The results from eleven cases are presented in table 1. One of the blood cultures, D, was taken from a case of pneumococcus meningitis, the rest of the samples were obtained from pneumonia patients. The identification of the P-C strains was made simply by noting the character of growth at 37° as shown in the last column of the table.

Of the eleven strains isolated, six showed definite lytic properties on blood agar at 37° as may be seen in the last column; and five grew normally without lysis at 37° as indicated at the bottom of the table. All of the strains gave large colonies of normal appearance at 25° as shown in the next to the last column. The number of cases so far studied is not large but these results serve to show that P-C variants occur in about 50 per cent of cases.

All of the P-C strains isolated from human sources were virulent for mice and all were recovered as P-C strains from the heart blood of the mice after death.

Besides the strains reported in the table, type I phantom colony variants were isolated at autopsy from a case of lobar pneumonia which had been shown by previous typing to be caused by a type I organism. Plating of the material from the pneumonic lung on blood agar at 37° gave no growth, but at 25° a scant growth which included three distinct colony types was obtained.

TABLE 1

Lytic characteristics of pneumococcus strains isolated from human cases of pneumococcus infection by cultivation at 25°C.

SOURCE	TYPE	CHARACTER OF GROWTH ON BLOOD AGAR AT 25°C. IN 3 DAYS	CHARACTER OF GROWTH ON BLOOD AGAR AT 37°C IN 36 HOURS
Blood A	IV	++ Large colonies, no lysis	- Complete lysis
Blood D	XII	++ Large colonies, no lysis	- Complete lysis
Blood G	?	++ Large colonies, no lysis	- Complete lysis
Sputum 1	V	++ Large colonies, no lysis	+ Colonies with irregular edges. Slight lysis
Sputum 4	?	++ Large colonies, no lysis	- Complete lysis
Sputum 5	III	++ Large colonies, no lysis	± Phantom colonies, marked lysis
Blood C	?	The last five strains here tabulated all gave a vigorous massive growth with no signs of lysis both at 25° and 37°	
Blood E	?		
Blood F	II		
Blood H	II		
Sputum 2	?		

- = no appreciable growth.

± = 50 to 200 colonies.

+ = over 200 distinct colonies

++ = massive growth.

Two of these grew normally at 25° but not at 37°. The third strain was extraordinary, in that it showed distinct lysis and formed phantom colonies even at 25°.

A few other such variants which show the lytic phenomena at 25° have been isolated but because of the methods used in isolation of the P-C variants, most of those so far studied grow

normally at 25°. Many of the freshly isolated cultures probably contain at least a few elements that grow normally at 37° and continued cultivation at this temperature selects the organisms which grow best and show the least tendency to lysis; thus, finally, a culture growing normally at 37° is produced.

MORPHOLOGICAL CHANGES PRODUCED BY THE ACTION OF THE
LYTIC PROCESS ON THE CELLS OF THE P-C VARIANTS

Morphological changes in the P-C variants grown on blood or serum agar have been observed by means of "klatch" preparations and by growing the organisms on agar in a hollow ground microscope slide and watching the development of the colonies, as described in the section on methods. Growth occurs normally until a few chains or a small colony is produced. Then a swelling of certain of the members of the chains occurs and this proceeds until part or all of the colony consists of swollen organisms. The swelling is followed by rupture and lysis of the cells so that finally a mass of gram-negative detritus is formed. Usually a nucleus of intact organisms, or at least one or two cells, will remain. These will develop to a normal colony if the culture is left at 25°, or may, after a long delay, grow up at incubator temperature to form a shining daughter colony on the dull surface of the original lysed colony.

The size and nature of the colonies formed depend on the speed of growth, the age of the culture at which the lytic process begins, and the rapidity with which it proceeds. Sometimes the organisms swell and break up soon after the culture is placed in the incubator. With other strains growth proceeds along with the process of lysis so that colonies showing extreme pleomorphism with balloon forms, bacillary forms, granular material, and only a few normal organisms result. These changes also occur to a less extent in some of the P-C strains when grown at 25°. In aerobic broth cultures grown at 37° a similar pleomorphism often occurs.

This process might be attributed either to autolysis or the action of the bacteriophage were it not for the fact, as shown later, that the lysis may be completely prevented by growing the cul-

ture under reduced oxygen tension or in the presence of carbon dioxide. There is no evidence that the granular material in the lysed colonies is viable. These colonies are often transplantable, but, when this is found to be the case, the presence of intact viable organisms cannot be ruled out. No attempts at filtration of these lysed cultures have been made.

COMPARISON OF P-C VARIANTS AND NORMAL STRAINS WITH REGARD TO BIOCHEMICAL CHARACTERISTICS

1. Growth on agar media under varying conditions of hydrogen ion concentration, carbon dioxide tension, and oxygen tension

The only cultural condition so far discussed that affects the lytic process in the P-C variants is temperature, but, as previously mentioned, pH, oxygen tension, and carbon dioxide tension have a marked effect on the lytic phenomenon and consequently determine whether a given P-C strain grows normally, forms small phantom colonies, or does not grow at all.

The effect of these factors and a comparison of the amount and type of growth obtained with normal and P-C strains under cultivation on blood agar at 37°, and glucose agar at 37° is presented in table 2. In the first column is noted the presence or absence of growth of normal and P-C strains at 37°C. under the standard conditions on blood agar of pH, 7.6 atmosphere tensions of oxygen (150 mm. of mercury) and carbon dioxide (less than 0.5 mm. of mercury). It will be seen that under these conditions only one of the P-C variants grows at all and this gives phantom colonies. Raising the tension of carbon dioxide to 40 mm. produces, as shown in the second column, a normal, vigorous growth of all the P-C variants except one. Tensions of carbon dioxide as low as 2 mm. of mercury will produce a considerable inhibition of lysis and consequent stimulation of growth of the P-C variants. For this reason the carbon dioxide produced by normal cultures growing in a closed space with the P-C variants is often sufficient to stimulate the growth of the latter and produce colonies of normal appearance. Valley and Rettger (1926, 1927) found that carbon dioxide in small amounts stimulates the

growth of a great many bacterial species on solid media and these observations have been confirmed by Walker (1932) for liquid media. In most cases the removal of carbon dioxide seems merely to prolong the lag phase, but, as has already been pointed out, the P-C variants of the pneumococcus when grown in the pres-

TABLE 2

Effect of oxygen, carbon dioxide and pH on growth and lysis, of P-C variants and normal strains on agar media at 37°C.

	BLOOD AGAR AT 37°C.								GLUCOSE AGAR AT 37°C.			
	7.6	7.6	7.6	7.6	7.6	7.1	6.9	6.4	7.6	7.6	7.6	7.6
pH →												
CO ₂ tension → mm. Hg.	<0.5	40	<0.1	<0.1	<0.2	<0.5	<0.5	<0.5	0.5	140	<0.5	30
O ₂ tension → mm. Hg.	150	110	0.5	8	40	150	150	150	150	110	5	5
Normal I s	++	++	-	++	++	++	++	++	±	-	++	+
I s 71	±P-C	++	-	++	-	-	-	-	-	±	-	+
I s 72	-	++	-	-	-	-	-	-	-	-	-	++
I s a 112	-	++	-	±P-C	-	-	-	-	-	-	-	-
I s a 134	-	++				-	-	-				
II s Normal	++	++		++	++	++	++	++	±	±	++	++
II s 21	-	++	-	-	-	-	+P-C	+	-	±	-	± or -
II s 241	-	-	±P-C	±	-	-	+P-C	-	-	-	-	-
IV s P-C	-	++	-	+P-C	-	-	+	-	-		-	±P-C

++ = Normal growth, no lysis.

± = Scant growth.

- = No growth, complete lysis.

P-C = Phantom colonies showing definite lysis

Origin and description of P-C strains as follows: I s 71, stock culture, virulent, rapid growing 25°; I s 72, stock culture, avirulent, slow growing 25°; I s a 112, direct from human autopsy, grows normally 25°; I s a 134, direct from human autopsy, phantom colonies at 25°; II s 21, stock culture, virulent, rapid growing 25°; II s 241, stock culture, avirulent, slow growing 25°; IV s P-C, direct from human blood grows normally 25°.

ence of the amounts of carbon dioxide ordinarily found in the atmosphere do not show any prolonged lag but undergo lysis and cease to grow after cell division has actually started. It was found that if the carbon dioxide is removed, by means of soda lime, as rapidly as it is formed no strain of pneumococcus will grow either at 25° or 37°.

The effect of reducing the oxygen tension on growth of the P-C variants on blood agar at 37° is shown in columns 3, 4, and 5 of table 2. One P-C strain grows normally at 8 mm. partial pressure of oxygen and less than 0.5 mm. of carbon dioxide, and in several others the lysis is somewhat inhibited so that instead of no visible growth, phantom colonies are produced. With the exception of one type II variant both normal and P-C strains fail to grow appreciably under conditions approaching a complete removal of oxygen as may be seen in the third column.

Although lowering the pH, as is indicated by the data recorded in the sixth, seventh and eighth columns of table 2, does produce some increase in the growth of the P-C variants, especially those of type II, the stimulation is much less than that produced by quantities of carbon dioxide too small to affect the pH of the medium appreciably. With large amounts of carbon dioxide the effect may be in part due to pH but in general the carbon dioxide seems to have a specific stimulating action on the growth of the P-C variants.

A comparison of the normal and P-C strains on glucose agar at 37° under atmospheric conditions, and with low oxygen tension, increased carbon dioxide tension, and both increased carbon dioxide and lowered oxygen is given in the last four columns of table 2.

None of the P-C variants will grow on 1 per cent glucose infusion agar at normal oxygen tension and 37°. The normal smooth cultures of types I and II do, however, grow moderately well on aerobic glucose agar, and the growth is greatly improved by lowering the oxygen tension to 5 mm.

It will be noted that carbon dioxide produces a slight improvement in the growth of several of the P-C variants on glucose agar but this improvement is not nearly as striking as that produced by a corresponding increase in carbon dioxide on the growth of P-C variants on blood agar. Lowering the oxygen tension does not by itself produce any improvement of the growth of the P-C variants on glucose agar but on blood agar a definite improvement in growth due to this change in cultural conditions was observed with three strains.

2. Effects of varying the constituents of blood agar media

The effect of varying the amount of blood cells and serum in infusion agar has been studied. The addition of more serum to the medium slightly improves the growth of some of the P-C variants. On a medium containing 1 per cent of laked cells but no serum a vigorous growth of the normal strains occurs. Many P-C strains on the other hand grow poorly or not at all on this medium at 37°. The use of hormone blood agar in place of infusion blood agar makes no difference in the growth of the P-C variants. Growth on chocolate agar, or Mueller's meatless medium, which contains 1 per cent peptone and 10 per cent of horse blood, is generally somewhat poorer than on blood agar.

3. Cultural conditions in broth media. Effect of pH and oxygen tension

Certain of the P-C variants, especially those that give visible colonies on blood agar at 37°, will grow normally in blood broth or horse-serum broth at 37° and pH 7.6, the reaction generally used in cultivation of the pneumococcus. On the other hand, a great many strains have been produced artificially and also isolated from cases of pneumococcus infection that grow slowly in broth or not at all under these conditions of temperature and pH. These also fail to grow on blood agar at 37°. Thus, if routine methods of isolation were employed their cultivation would be very difficult, or impossible. Studies of growth in broth at various degrees of acidity show that the P-C variants have a pH growth range different from the normal forms. The results of these experiments are summarized in table 3. None of the P-C variants will grow on blood broth above pH 7.8 but all normal-growing strains grow just as well at pH 8.2 as they do at 7.6. The acid death point seems to be about the same in all cases.

Sealing the tubes of blood broth with vaseline before incubation makes no difference in the growth of the P-C variants but with glucose broth sealing the tubes makes a great difference in the amount of growth obtained. None of the P-C variants will grow aerobically on old glucose broth but some give a very slight growth on freshly prepared or recently boiled aerobic broth after

forty-eight hours at 37°. Lowering the pH to 7.0 also improves the growth of some of the strains.

Most of the P-C variants grow well in recently boiled 1 per cent glucose broth sealed with vaseline; but a longer period of incubation at 37° is required than is necessary with normal strains. Under these conditions the pH growth-range is similar to that on blood broth but is somewhat narrower as may be seen in the last two columns of table 3.

TABLE 3

Growth pH range in aerobic and vaseline sealed blood broth and glucose broth

STRAIN*	pH RANGE OF GROWTH AT 48 HOURS			
	Blood broth aerobic	Blood broth sealed	Glucose broth aerobic	Glucose broth sealed
IS normal	8.4-6.1	8.4-6.1	8.4-7.0	8.4-6.6
IS 71	7.8-6.1	7.8-6.1	7.0 irregular	7.8-6.8
IS 72	7.2-6.1	7.2-6.1	No growth	7.8-7.4
ISa 111	6.8-6.1	6.8-6.1	No growth	
IIS normal	8.4-6.1	8.4-6.1	8.4-6.6	8.4-6.6
IIS 21	7.6-6.1	7.6-6.1	7.4-7.0 irregular	7.8-6.6
IIS 241	No growth	No growth	No growth	No growth

* Origin and description of PC strains as in table 2.

IS 71, stock culture, virulent, rapid growing 25°.

IS 72, stock culture avirulent slow growing 25°.

ISa 111, avirulent dissociant of strain from human autopsy, grows normally 25°.

IIS 21, stock culture, virulent, rapid growing 25°.

IIS 241, stock culture avirulent, slow growing 25°.

REVERSION OF THE P-C VARIANTS TO NORMAL FORMS

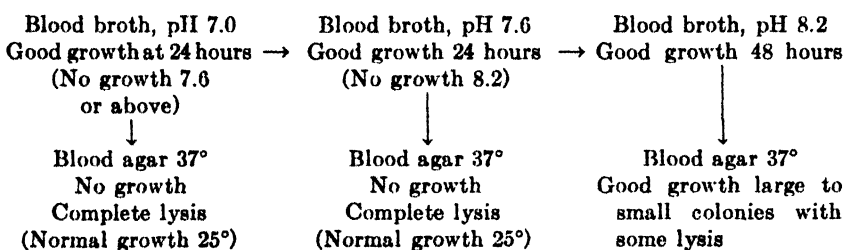
The P-C variants show great differences in stability. Strains that give some growth on blood agar at 37° may be considered to contain a few normal-growing elements and these may be changed back to normal-growing cultures after several generations at incubator temperature and selection of the largest colonies. If the culture contains many normal forms along with the P-C, the normal forms stimulate the P-C variants by the carbon dioxide they produce, when grown on solid media at 37° in a closed space. In liquid media at 37° the normal forms if originally present in

the culture tend to overgrow the P-C variants, especially in broth above pH of 7.6.

P-C variants that grow at 37° in blood broth at pH 7.6 but not on blood agar may be carried unchanged in blood broth for many generations before any sign of reversion appears. If broth of pH 7.0 is used the rate of reversion is still slower. In general the more alkaline the medium the sooner the appearance of normal growing forms in the culture.

With some strains a rapid reversion to normal can be brought about by growing the P-C variant first in blood broth at pH 7.0 and then transferring a drop of this broth culture at twenty-four hours to blood broth at pH 8.2. Growth may be delayed in the alkaline blood broth but when it occurs many normal forms are obtained on plating to blood agar. The more stable P-C strains cannot be caused to revert in this manner in two generations but the same thing may be accomplished by several transfers in broth of gradually increasing pH until growth in the alkaline broth occurs.

An outline of this procedure is shown in the diagram given below.



The various colony forms which grow out of the reverted strains seem to represent definite stages in the reversion process and in certain cases stable strains each representing one of these stages have been isolated. The colony forms on blood agar at 37° observed in these various stages of reversion are:

- (1) Small phantom colonies; marked lysis.
- (2) Medium irregular colonies; slight lysis.
- (3) Large normal growing colonies; no lysis.

The first two types revert rather easily to the forms which give

no appreciable growth on blood agar at 37°. The last type represents the stable, vigorously-growing form ordinarily found in stock cultures.

Type II P-C strains are exceptional in that they revert to normal growing forms on mouse passage and also in blood broth even in a single generation without change in pH. This abrupt reversion has not been noted in the case of pneumococcus P-C strains of types I, III or IV.

Another type of reversion occurred in the type III P-C variant isolated from pneumonia sputum (see table 1). After several generations on blood agar at 25° this strain began to form opaque patches in the transparent colonies. These opaque patches were found to contain a dissociant which grew normally at 37°, and was very similar to the stock culture of type III. The same type of reversion in normal-growing forms (as observed in the case of type III, by daughter colony dissociation at 25°) was also found to occur in another strain of unknown type isolated from pneumonia sputum.

In general the reverted strains are similar in virulence and antigenic composition to the P-C strains from which they are derived. It is evident, therefore, that the change from normal to P-C and back again to normal may be accomplished without any appreciable alterations in the other characteristics of the pneumococcus. As with the transformation of normal to P-C so also with the reversion of P-C to normal, methods which involve prolonged cultivation, aging, or dissociation of the cultures will produce changes in virulence and antigenic composition in addition to causing the appearance or disappearance of lytic properties.

COMPARISON OF P-C AND NORMAL STRAINS WITH REGARD TO VIRULENCE AND ANTIGENIC COMPOSITION

As stated in the previous section, stimuli which cause S to R dissociation, such as prolonged cultivation without animal passage, or growth in antiserum, will cause a reduction in virulence or the appearance of R forms in the P-C variants just as they do in the normal-growing forms. P-C variants showing all degrees of virulence have been isolated, so that it is evidently meaningless

to speak of the virulence or avirulence of P-C variants as a general class.

P-C variants derived by the daughter colony dissociation from a smooth strain behave exactly like the normal smooth forms in that their virulence may be raised by mouse passage or lowered by prolonged cultivation on blood agar. Rough P-C variants can-

TABLE 4
Virulence and antigenic composition of P-C variants

STRAIN*	MAXIMUM DILUTION WHICH KILLS MICE 0.2 CC. 24 HOUR BLOOD BROTH CULTURES	TIME OF DEATH OF MICE (AVERAGE) WITH MAXIMUM DILUTIONS	MAXIMUM DILUTION† OF AUTO-LYSATE WHICH GIVES PRECIPITATE WITH HOMOLOGOUS ANTISERUM	TYPE SPECIFICITY AS DETERMINED BY AGGLUTINATION
Is normal	10^{-6}	1-2 days	1:160	Type I
Is 71	10^{-6}	1-2 days	1:120	Type I
Is 712	Undiluted	1 day	1:160	Type I
Is 72	—	Survived	1:160	Type I
Is 732	—	Survived		None, R
II s normal	10^{-4}	1 day	Not done	Type II
II s 122	10^{-4}	6-12 days	Not done	Type II
				Slight agglutination I and III
II 241	—	Survived	0	None, R
III s normal	10^{-6}	1 day	1:100	Type III
III s P-C	10^{-6}	1 day	1:100	Type III

* All strains designated by numbers are P-C variants.

Is 71, 72, II s 241 are the same strains as those shown in table 3.

Is 712; a dissociant of Is 71.

Is 732; a rough P-C variant from the strain Is 71.

II s 122; a smooth dissociant from stock type II culture.

† The autolysates were boiled successively at pH 3.5 and 8.5 and the coagulum removed (see section on methods). The precipitates obtained with the antisera were considered to be due to the specific soluble substance.

not be obtained directly from normal growing rough cultures and must therefore be produced from the smooth P-C strains.

In table 4 are presented the results of comparative studies on the virulence and antigenic composition of normal-growing smooth strains and P-C variants of various degrees of virulence for mice. The first column gives the results of virulence titra-

tions in mice using 0.2 cc. of various dilutions of twenty-four-hour blood broth cultures. Studies on the antigenic composition were confined to tests for the specific soluble substance by means of precipitin titrations on broth culture autolysates (column 2), prepared as described in the section on methods, and by means of agglutination tests on broth suspensions of pneumococcus with homologous and heterologous sera (column 3).

These results show that the P-C variants can equal the normal-growing strains in virulence. Some of the P-C strains show a decreased virulence with a retention of the other smooth characteristics. This diminished virulence is evident in two ways; first, by a decreased titre for mice, as with strain I S 712; second, by an increase in the length of the time required to kill mice as is strikingly shown with the strain II S 122.

From the results presented in the last two columns of table 4 it may be seen that the virulent, and certain of the avirulent, P-C variants of type I form as much specific soluble substance as do the normal-growing forms. Strain I S 712 has all of the typical smooth characteristics except high virulence for mice and strain I S 72 is smooth by antigenic tests but avirulent by the mouse test.

The virulent P-C variant of type III mentioned in table 4 was found by precipitin tests with the autolysate to form the same amount of specific carbohydrate as the normal-growing strain. No avirulent P-C variants of type III have as yet been isolated. Further studies on the relations between the antigenic composition, the virulence, and the colony form of normal and P-C variants are now in progress.

DISCUSSION

Definite evidence has been presented in this paper that the P-C variation as characterized by the tendency of the P-C strains to lyse at 37°C., represents a change that is entirely independent of the alterations in antigenic composition which occur in the smooth-to-rough transformation. Additional examples of independent variations in the properties of pneumococcus cultures have been observed during the course of the work and will

be reported later. Nungester (1933) has recently called attention to this independent variation of the properties in other bacterial species. On the basis of the evidence at hand we believe that the conception of a single change from rough to smooth or smooth to rough with intermediate forms is too limited and is not in accord with experimental observations on the variations brought about by daughter colony dissociation. This method as employed in the course of this work did not produce a smooth to rough transformation.

The P-C variants when grown under certain conditions resemble both in colony form and morphology the G forms of Hadley (1931), who considered these G forms to represent a filterable stage in a life cycle. Attempts to show whether or not the lysed P-C variants represent a filterable stage in a life cycle by filtration experiments or serial plate washings, as described by Hadley, have not been made. In the case of the P-C variants a further study of the nature of the lytic process itself is in our opinion more important than attempts at filtration. As shown in the present work the colony form and morphology of the P-C variants is due to the operation of a lytic process which may be inhibited by appropriate alterations in temperature, pH, and oxygen and carbon dioxide tensions; under the changed environmental conditions the P-C strains produce colonies identical with those of normally growing pneumococcus strains. A further elucidation of the chemical nature of lytic processes should lead to a definite conclusion as to whether they represent changes from non-filterable to filterable forms in a life cycle, or an irreversible destructive chemical process similar to the autolysis of dead cells.

A much more detailed knowledge of the metabolism of living cells will be required before any conclusion may be drawn as to the chemical nature of the lytic processes which occur in the P-C variants of the pneumococcus on blood agar at 37°. The effect of carbon dioxide in preventing the lysis is very striking but as yet no hint as to its mode of action has been obtained. The fact that the P-C variants grow well at 25° but not at 37° indicates that some destructive chemical reaction in the P-C cells is speeded up by raising the temperature, and this reaction is inhibited by carbon dioxide and to a less extent by lowering the pH.

In some cases the reduction of oxygen tension or the production of more strongly reducing conditions in the medium inhibits the lytic process. This latter observation indicates that the lytic process is connected with an oxidative mechanism in the cell. No transmissible lytic agent has been demonstrated in the filtrates of P-C variant cultures. The lytic action of the bacteriophage is not readily affected by changes in temperature, pH, and CO₂ tension as is the lytic process in P-C variants.

P-C variants have been frequently isolated from blood, sputa, and the lungs of pneumonia patients. This is not surprising in view of the fact that conditions in the body are nearly optimal for the production and growth of these variants. In the living body the low oxygen tension, high CO₂ tension and pH near 7.3 favor the appearance of the P-C variation whereas the high oxygen tension, minimal CO₂ tension, and pH of 7.6 or above occurring under conditions of artificial cultivation lead to the disappearance of the P-C variants and the maintenance of those organisms which exhibit the cultural characteristics of the typical pneumococcus. At present nothing is known concerning the significance of the demonstration of P-C variants in cases of pneumonia. It is possible that they may be derived in the body from the so-called normal strains and therefore be of minor importance. On the other hand they may represent the primary infective agent in pneumonia. The unusual cultural properties of the P-C variants and the attendant difficulties in isolating and cultivating them by routine methods might lead to errors and misconceptions in the experimental study of pneumococcus infection. For this reason a further study of the rôle of P-C variants in disease is indicated.

SUMMARY

Methods are described for the isolation from human sources and the artificial production of stable strains of the pneumococcus which undergo rapid lysis or fail to grow under the ordinary conditions at 37°. Strains showing such characteristics have been termed phantom colony variants or P-C variants.

The effects of cultivation at 25°, carbon dioxide, pH, and oxygen tension on the growth and lysis of P-C variants, are described.

The P-C variant strains are compared with normal forms as regards growth requirements, virulence and antigenic composition.

Evidence is adduced that the P-C variation is a change independent of the ordinary smooth-to-rough variation.

Methods are described for causing reversion of the phantom colony variants to normal-growing smooth forms by cultivation in alkaline media, and under certain other conditions.

The direct isolation of the phantom colony variants from cases of human infection indicates further study of their possible rôle in disease.

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STUDIES ON THE LEUCOCYTE CONTENT OF MILK DRAWN FROM BRUCELLA ABORTUS INFECTED UDDERS¹

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The significance of the presence of *Brucella abortus* in the udder of the dairy cow is a matter of controversy. Certain investigators have presented evidence to show that pathological changes of the udder tissue accompany the growth of this organism within the udder. Others have contended that the growth of *Br. abortus* within the udder does not appear to produce harmful results. In a study in which leucocyte counts were made on a large number of milk samples drawn from both *Br. abortus* infected and non-infected animals, little difference was noted in the cellular content of milk from these two classes of animals. Since a number of the infected animals had a high agglutination titer for *Br. abortus* in both the blood and milk serum and regularly produced milk of a low leucocyte content, it appeared advisable to study the leucocyte content of the milk from the reacting animals in relation to the presence of this organism in the producing quarter.

REVIEW OF LITERATURE

Cooledge (1918) found the milk from udders infected with *Br. abortus* to have an average cell count of 4,800,000 per cubic centimeter, this being five times greater than the cell count of milk drawn from apparently normal udders. He found that udders artificially infected with this organism were quick to show an increase in the cellular count. From his study, he concluded that *Br. abortus* infections accounted for many of the samples

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of milk having high cellular counts as drawn from apparently normal udders. Tweed (1923) reported an average cell count of 1,558,000 per cubic centimeter for milk drawn from *Br. abortus* infected udders as compared to 626,000 per cubic centimeter for milk from non-infected animals.

Hallman, Sholl and Delez (1928), Sholl and Torrey (1931), and Runnells and Huddleson (1925) obtained evidence by histological studies of *Br. abortus* infected udders that pathological changes in the udder tissue were associated with infections of this organism. Hallman, Sholl and Delez (1928) stated:

It is common knowledge that mastitis is of considerable clinical importance in *Bact. abortus* infected herds. Whether primary invasion of the udder by *Bact. abortus* bears any relation to this we do not know. Our researches indicate quite clearly to us that we can no longer assume that *Bact. abortus* infection of the udder is without harmful effects. We shall not be surprised if future researches reveal that it is in the udder that *Bact. abortus* assumes its greatest economic importance.

Bang and Bendixen (1931) found no visible alteration of the milk or udder to occur even when the organisms were present up to 30,000 per cubic centimeter. When present in large numbers, they found disturbances of secretion such as are seen in latent infections by other bacteria. They observed the posterior glands to be most frequently infected.

Smith *et al.* (1923) concluded that *Br. abortus* multiplied in the residual milk in the acini and ducts rather than in the udder tissue and that the presence of this organism in the udder did not result in pathological changes. Buck (1927) stated:

The abortion bacilli, when present do not appear to produce harmful results in the udder. The multiplication of the germ in this organ seems to be merely a provision of nature for the perpetuation of the infection during those intervals when conditions are unfavorable for growth of the germs in the uterus.

Recent studies made by a number of investigators concerning the cell count of milk drawn from normal healthy udders have thrown considerable doubt upon the results reported by Cooledge

(1918) and Tweed (1923) in regard to the average leucocyte count of milk drawn from apparently normal udders. Cherrington, Hansen, and Halversen (1933) concluded that milk from normal udders usually contains less than 50,000 cells per cubic centimeter, whereas milk from animals suffering with mastitis almost invariably contains more than 100,000 per cubic centimeter. Hucker (1933) found that no quarter free from fibrotic tissue or indurations produced milk having a cell count in excess of 150,000 per cubic centimeter. He concluded that udder quarters producing milk having cell counts of more than 150,000 per cubic centimeter should be considered as suspicious, if not definitely proved to be infected with streptococci. In a study made in this laboratory, Prouty (1934) found that approximately 60 per cent of the samples having cell counts ranging from 250,000 to 500,000 per cubic centimeter contained excessive amounts of catalase, thus indicating the presence of abnormal udder condition. Based on the catalase test, 98 per cent of the samples used in the above study having cell counts ranging from 750,000 to 1,000,000 were from abnormal or mastitis udders.

METHODS

The data to be presented were obtained from the milk of 18 *Br. abortus* infected animals. All of the animals had exhibited positive blood agglutination reactions for a period of two years or longer. Milk from cows in the advanced stages of lactation, the last three months of the lactation period, or those which had just freshened was excluded from this study for the reason that such milk often has an increased leucocyte count due to physiological rather than to pathological changes in the udder.

In a number of instances pronounced evidence of the presence of streptococcic mastitis, as shown by blood-agar plate cultures, decreased hydrogen ion concentration, and increased catalase content of the milk was observed, thus accounting in part for several of the high leucocyte counts obtained.

Samples of the fore milk were collected aseptically from each quarter into sterile containers after the initial four or five streams had been discarded. They were examined immediately.

TABLE 1

Leucocyte count of milk in relation to the presence of Br. abortus and to the agglutination titer of milk and blood serum

ANIMAL NUMBER	QUARTER	AGGLUTINATION TITER		BR. ABORTUS	TIMES COUNTED	AVERAGE CEL- LULAR COUNT PER CUBIC CENTIMETER
		Blood	Milk			
59	R F	cc. 0.004	cc. 0.08	—	10	84,000
	R H		0.04	—	10	215,000
	L F		0.04	+	10	56,000
	L H		0.04	—	10	144,000
72	R F	0.02	Negative	—	13	91,000
	R H		Negative	—	13	1,162,000*
	L F		Negative	—	13	85,000
	L H		Negative	—	13	76,000
82	R F	0.004	Negative	—	7	661,000
	R H		Negative	—	7	86,000
	L F		0.01	—	7	7,480,000*
	L H		0.01	+	7	203,000
88	R F	0.004	0.02	+	14	225,000
	R H		0.02	+	14	283,000
	L F		0.02	+	14	246,000
	L H		0.02	+	14	2,900,000*
94	R F	0.02	Negative	—	7	80,000
	R H		Negative	—	7	68,000
	L F		Negative	—	7	114,000
	L H		Negative	—	7	76,000
97	R F	0.02	Negative	—	4	208,000
	R H		Negative	—	4	70,000
	L F		Negative	—	4	606,000
	L H		Negative	—	4	75,000
1028	R F	0.004	0.08	—	6	118,000
	R H		Negative	—	6	967,000
	L F		0.08	—	6	303,000
	L H		0.08	—	6	113,000
1030	R F	0.004	Negative	—	5	150,000
	R H		0.01	—	5	130,000
	L F		0.01	+	5	210,000
	L H		0.08	—	5	66,000
1034	R F	0.02	Negative	—	4	290,000
	R H		Negative	—	4	125,000
	L F		Negative	—	4	383,000
	L H		Negative	—	4	63,000

TABLE 1—*Concluded*

ANIMAL NUMBER	QUARTER	AGGLUTINATION TITER		BR. ABORTUS	TIMES COUNTED	AVERAGE CEL- LULAR COUNT PER CUBIC CENTIMETER
		Blood	Milk			
1046	R F	cc 0 004	cc. 0 02	+	9	86,000
	R H		0 02	+	9	53,000
	L F		0.04	+	9	91,000
	L H		0 02	+	9	78,000
2035	R F	0 004	0 04	—	8	165,000
	R H		0.02	+	8	107,000
	L F		0.04	—	8	141,000
	L H		0.02	—	8	295,000
2052	R F	0 004	Negative	—	8	190,000
	R H		Negative	—	8	110,000
	L F		Negative	—	8	276,000
	L H		Negative	—	8	350,000
2053	R F	0 004	0 04	—	5	44,000
	R H		0 01	+	5	250,000
	L F		0 04	—	5	62,000
	L H		0.02	—	5	36,000
2074	R F	0 01	0 04	+	8	67,000
	R H		0 04	—	8	333,000
	L F		0 04	—	8	40,000
	L H		0.04	—	8	80,000
2075	R F	0 004	0 01	—	11	243,000
	R H		0 01	+	11	1,710,000*
	L F		0 01	+	11	178,000
	L H		0.02	—	11	200,000
2077	R F	0 004	0 08	—	3	115,000
	R H		0 04	—	3	48,000
	L F		0 04	+	3	22,000
	L H		0.04	+	3	85,000
3011	R F	0 004	0 08	—	8	402,000
	R H		0.01	+	8	385,000
	L F		0.08	—	8	352,000
	L H		0.08	—	8	148,000
3013	R F	0.004	0 08	—	7	60,000
	R H		0.02	+	7	117,000
	L F		0.08	—	7	52,000
	L H		0.02	+	7	127,000

* Mastitis caused by streptococci.

Leucocyte counts were made by the direct microscopic method developed by Prescott and Breed (1911). Newman's (1927) formula No. 2 was used in staining the preparations. Twenty fields of each preparation were counted.

The presence of *Br. abortus* in the udder was determined by culturing the aseptically drawn milk on cooked-blood-agar plates as described by Henry, Traum and Haring (1932). Typical colonies were further identified by means of cultural and serological tests. All udder quarters reported as negative in table 1 were recorded as such only after being cultured at least five times over a period of several months' duration.

The agglutination titer of both the blood and milk serum was determined by means of the rapid macroscopic method of Huddleson and Carlson (1926). Antigen secured from the Jensen-Salsbery Laboratories, Inc., Kansas City, Missouri, was used in these determinations. A circular prepared by the Jensen-Salsbery Laboratories and enclosed with the antigen stated that the amounts of serum 0.08, 0.04, 0.02, 0.01 and 0.004 cc. correspond approximately to the following respective dilutions as used in the tube method: 1:25, 1:50, 1:100, 1:200, and 1:500.

PRESENTATION OF DATA

The average leucocyte counts of milk drawn from individual udder quarters in relation to the presence of *Br. abortus* and the agglutination titer of both the milk and blood serum are shown in table 1. Under the agglutination titer is recorded the smallest amount of serum showing complete agglutination of the antigen.

DISCUSSION OF RESULTS

Thirteen of the 18 cows used in this study were found by cultural methods to harbor *Br. abortus* in one or more quarters of the udder. Only two animals were found to be infected in all four quarters. Seven were found to eliminate *Br. abortus* from only one quarter. Twenty-one of the 72 quarters were found to be infected.

The average leucocyte count per cubic centimeter of milk from the 21 infected quarters was 355,000 as compared to 343,000

for milk from the 51 quarters that gave negative cultural findings. During the period over which this study extended animals 88, 2075, 72 and 82 each suffered from streptococcic mastitis in one quarter. Maximum leucocyte counts for milk from these respective udder quarters were 9,500,000; 3,600,000; 4,000,000 and 14,000,000 per cubic centimeter. When the cell counts of the samples of milk drawn from the left hind quarter of cow 88 and the right hind quarter of cow 2075 were excluded in computing averages, an average cell count of 145,000 was obtained for the milk samples drawn from the remaining 19 *Br. abortus* infected quarters. Likewise, when the milk from the right hind quarter of cow 72 and the left front quarter of 82 was omitted, an average cell count of 185,000 per cubic centimeter was found for the milk drawn from the 49 remaining quarters which gave negative findings when cultured for *Br. abortus*.

In a previous study of the leucocyte content of milk a large number of samples were from animals which periodic agglutination tests had shown to be abortion free. Three hundred and ninety-six samples from 14 animals gave an average cell count of 225,000 per cubic centimeter. Several of the animals of this herd, during the time in which they were under observation, showed pronounced positive evidence of mastitis in one or more udder quarters and when the milk from these quarters was omitted an average leucocyte count of 177,000 per cubic centimeter was found for the remaining samples. No significant differences, therefore, were seen to exist between the leucocyte counts of milk drawn from *Br. abortus* infected udders and of milk from animals free from this disease.

Five of the animals giving positive blood agglutination reactions produced milk in all quarters that contained no agglutinins for *Br. abortus* and in no instance was *Br. abortus* isolated from the milk of these animals. All samples of milk giving negative agglutination reactions in amounts of milk serum of less than 0.08 cc. yielded negative cultural findings for *Br. abortus*. This organism was isolated from five of the 13 quarters in which 0.04 cc. of milk serum was necessary to produce agglutination reactions and from 16 of the 22 quarters giving positive agglutination reactions in 0.02 cc. or less of milk serum.

SUMMARY AND CONCLUSIONS

A study was made of the leucocyte content of the milk from 18 abortion-infected cows in relation to the presence of *Br. abortus* within the udder.

Thirteen cows were found by cultural methods to harbor *Br. abortus* in one or more quarters of the udder. Two were found to be infected in all quarters and seven in only one quarter. Twenty-one of the 72 quarters were found to be infected.

The average leucocyte count per cubic centimeter of milk from the 21 infected quarters was 355,000 as compared to 343,000 for milk from the 51 quarters that gave negative cultural findings. Two of the *Br. abortus* infected quarters and two of the non-infected quarters produced milk of a high leucocyte count due to the presence of active streptococcic mastitis. When the samples of milk coming from these quarters were omitted in computing averages, average cell counts of 145,000 and 185,000 per cubic centimeter were obtained for the samples from the *Br. abortus* infected and non-infected quarters respectively. Similar average leucocyte counts were obtained for samples of milk from animals in an abortion free herd.

All samples of milk giving negative agglutination reactions in amounts of milk serum less than 0.08 cc. gave negative cultural findings for *Br. abortus*.

The results of the present study showed no significant differences in the leucocyte count of milk from *Br. abortus* infected udders and of milk from animals free from this disease.

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THE PHYSIOLOGICAL YOUTH OF A BACTERIAL CULTURE AS EVIDENCED BY CELL METABOLISM¹

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OBJECTIVES

In an earlier contribution from this laboratory (Walker and Winslow, 1932) it was shown that toward the end of the initial lag period in either peptone or lactose-peptone water, there is manifest an enormous increase in metabolic activity, particularly with respect to ammonia production. Formation of CO₂ per cell per hour is increased thirty to seventy fold and formation of NH₃ nitrogen fifty to one-hundred-and-fifty fold as compared with the peak stability rates. There is, in these increases, a very clear demonstration of the physiological youth of the bacterial cells as postulated by Sherman and Albus (1923).

These phenomena seemed so important as to warrant further and more detailed study under different growth conditions and with observations made at more frequent intervals during the course of the growth cycle.

METHODS

The culture employed was the same strain of *Esch. coli* used in previous studies in this laboratory. The media for the growth and metabolism experiments were peptone-water (1 per cent Difco Bacto peptone) and glucose-peptone water (0.5 per cent Baker's c.p. glucose plus 1 per cent Difco Bacto peptone). Starting at a

¹ This study was assisted by a grant from the Research Fund of the Yale School of Medicine. The data on ammonia production are from an essay presented by Evelyn Huntington in partial fulfillment of requirements for the Certificate in Public Health of Yale University.

pH of 6.9 to 7.2 one series of studies on CO_2 production and one on ammonia production was conducted with each of these media under continuous aeration with air which had been freed from CO_2 and NH_3 . The aeration train used for this purpose was the one described by Walker (1932). A fifth series of experiments on NH_3 production was made without aeration, in the first medium (peptone water) only.

Prior to inoculation of each test medium the organism was washed off from twelve to eighteen hour agar slants with sterile water, filtered through paper to remove clumps, diluted appropriately and added to the desired medium in Dreschel bottles so as to give an average initial count of 10 to 20 million organisms per cubic centimeter. Two duplicate culture bottles and a third uninoculated control bottle were placed in a water bath at 37°C . and aerated for at least thirty minutes to ensure stable conditions before inoculation. The duration of periodic observations after inoculation was five hours, which carried the culture well toward, or slightly past, the end of the phase of logarithmic increase. In the NH_3 experiments the culture was plated for cell counts and chemical determinations made every half hour (in the CO_2 studies, every hour).

In the three series of NH_3 studies, $\text{NH}_3\text{—N}$ formed in the medium was determined by analysis of the medium only, since our earlier work had shown that the amount of ammonia carried over in the aeration train is negligible in the early phases of the life cycle (Walker and Winslow, 1932). The $\text{NH}_3\text{—N}$ was determined by the Van Slyke and Cullen modification of Folin's aeration method with the minor changes suggested by Walker (Walker and Winslow, 1932).

In the two series of studies of carbon dioxide, we determined both the carbon dioxide in the medium and that carried off in the aeration train, the former figure being obtained by the use of the fine-bore blood gas apparatus described by VanSlyke and Stadie (1921), the latter by absorption in standard $\text{Ba}(\text{OH})_2$ in a modified form of the Brady-Meyer² tube used by Walker and Winslow (1932). All

² Changes had to be made in the Brady-Meyer absorption tubes in the present studies in order to accomplish reasonably complete detention of the small amounts

quantitative analytical determinations for CO_2 and NH_3 throughout all analytical sampling periods were applied simultaneously to the cultures and to the uninoculated control medium and the yields reported represent the excess over the mean of all the control determinations of a given experiment.

The yield of ammonia or carbon dioxide per cell per hour during a given period was computed by the formula of Buchanan (1918) as used by Walker and Winslow (1932).

$$r = \frac{P (\log b - \log B)}{0.434 (b - B) t}$$

where r = the amount of product per cell per hour

t = duration of period in hours

P = total product formed during time t

B = number of bacteria at start of time t

b = number of bacteria at end of time t

This formula was designed to estimate the effective population during the period of logarithmic increase and is more accurate for that period than a formula based on the mean of initial and final

of CO_2 aerated off in single early hours. To this end, special tubes were constructed with the bulbs considerably smaller than those of the ordinary Brady-Meyer tube. The stem was also lengthened to fit into a $8 \times 1\frac{1}{4}$ inch test tube instead of the usual Erlenmeyer flask. For about 5 to 6 cm. from the bottom this stem was enlarged into a cylindrical chamber of 1 cm. diameter, with slightly increased bulbous portions at top and bottom and a small hole at the bottom. This stem was not pushed to the extreme end of the enclosing test tube. Dimensions of the whole assembly were then such that when 20 cc. was the volume of standard $\text{Ba}(\text{OH})_2$ employed, about one third of the absorbent remained in the bottom of the test tube, one third occupied the cylindrical enlargement of the Brady-Meyer stem and the remaining third passed up into the succession of small bulbs making up the true Brady-Meyer part of the apparatus. The inlet from the culture to the absorption assembly was allowed to dip into the third of the $\text{Ba}(\text{OH})_2$ remaining in the bottom of the large test tube. Thus CO_2 in the air current from the culture was subjected to a first absorption as it bubbled from the inlet up through the absorbent in the test tube, a second absorption as it passed along the surface of this fluid in order to enter the stem of the Brady-Meyer tube, a third absorption as it passed up the cylindrical chamber of the stem, and a final absorption as it was scrubbed through the bulbs of the Brady-Meyer tube proper. Hence, with this set-up, four serial extractions were achieved with a total absorbent of only 20 cc., which was easily rinsed down into and titrated in the single large test tube after use.

population. For the lag period it gives—so far as our data are concerned—results essentially identical with those of the simpler method of using the mean between initial and final count, and has therefore been employed throughout all the hourly or half-hourly observation periods.

GENERAL RESULTS

The general results of our experiments are summarized in tables 1 to 5. Each figure in each of these tables is the average of eight independent duplicate experiments. These duplicate experi-

TABLE 1
Growth and ammonia yield in aerated peptone water

AGE	BACTERIA, MILLIONS PER CUBIC CENTIMETER		NH ₃ N YIELD		
	Actual	Log	Increment	Cumulative total	Rate per cell per hour during previous interval Mgm $\times 10^{-11}$
<i>hours</i>			<i>mgm. per 100 cc.</i>	<i>mgm. per 100 cc.</i>	
0	14.5 \pm 2.8	1.16			
0.5	15.2 \pm 2.6	1.18	0.06 \pm 0.04	0.06	7.9
1.0	15.2 \pm 2.6	1.18	0.09 \pm 0.05	0.15	11.8
1.5	16.3 \pm 4.2	1.21	0.40 \pm 0.06	0.55	50.0
2.0	22.8 \pm 6.5	1.36	0.10 \pm 0.10	0.65	10.6
2.5	55.1 \pm 14.2	1.74	0.10 \pm 0.14	0.75	4.0
3.0	92.3 \pm 23.3	1.97	0.21 \pm 0.12	0.96	6.0
3.5	216.0 \pm 54.2	2.33	0.14 \pm 0.12	1.10	1.9
4.0	333.0 \pm 73.3	2.52	0.34 \pm 0.11	1.44	2.5
4.5	582.0 \pm 141.9	2.76	0.46 \pm 0.08	1.90	2.0
5.0	727.0 \pm 173.7	2.86	0.50 \pm 0.26	2.40	1.6

ments showed, in general, a reasonably satisfactory degree of uniformity, for phenomena of the type studied. The probable errors of the means, as cited in the tables for bacterial counts, are less than one-fourth of the recorded mean values in all but two instances and are generally far below this proportion, which is by no means bad for independent duplicate growth curves. The probable errors of the mean determinations of carbon dioxide are relatively lower still, (one sixth or less of the mean) except in the case of the results for the first hour. The ammonia data are

TABLE 2

Growth and ammonia yield in aerated glucose-peptone water

AGE	BACTERIA, MILLIONS PER CUBIC CENTIMETER		NH ₃ N YIELD		
	Actual	Log	Increment	Cumulative total	Rate per cell per hour during previous interval mgm. $\times 10^{-11}$
<i>hours</i>			<i>mgm. per 100 cc.</i>	<i>mgm. per 100 cc.</i>	
0	10.4 \pm 1.8	1.02			
0.5	10.1 \pm 1.5	1.00	0.04 \pm 0.01	0.04	5.9
1.0	13.6 \pm 3.0	1.13	0.02 \pm 0.00	0.06	5.1
1.5	15.7 \pm 3.9	1.20	0.17 \pm 0.02	0.23	26.2
2.0	30.9 \pm 12.4	1.49	0.27 \pm 0.06	0.50	24.6
2.5	70.8 \pm 16.7	1.85	0.16 \pm 0.06	0.66	6.2
3.0	145.0 \pm 41.5	2.16	0.01 \pm 0.03	0.67	0.4
3.5	287.0 \pm 66.8	2.46	0.04 \pm 0.03	0.71	0.3
4.0	538.0 \pm 43.0	2.73	0.08 \pm 0.05	0.79	0.3
4.5	703.0 \pm 71.5	2.85	0.11 \pm 0.07	0.90	0.4
5.0	890.0 \pm 51.3	2.95	0.23 \pm 0.03	1.13	0.6

TABLE 3

Growth and ammonia yield in unaerated peptone water

AGE	BACTERIA, MILLIONS PER CUBIC CENTIMETER		NH ₃ N YIELD		
	Actual	Log	Increment	Cumulative total	Rate per cell per hour during previous interval mgm. $\times 10^{-11}$
<i>hours</i>			<i>mgm. per 100 cc.</i>	<i>mgm. per 100 cc.</i>	
0	10.9 \pm 3.8	1.04			
0.5	10.4 \pm 1.2	1.01	0.02 \pm 0.00	0.03	5.8
1.0	10.0 \pm 1.6	1.00	0.05 \pm 0.00	0.07	7.8
1.5	9.7 \pm 0.9	0.99	0.16 \pm 0.05	0.23	31.9
2.0	16.0 \pm 3.3	1.20	0.34 \pm 0.11	0.57	54.0
2.5	34.0 \pm 4.4	1.53	0.10 \pm 0.03	0.67	8.4
3.0	62.8 \pm 7.2	1.80	0.09 \pm 0.02	0.76	3.4
3.5	87.5 \pm 8.0	1.94	0.10 \pm 0.09	0.86	2.7
4.0	103.0 \pm 11.9	2.01	0.31 \pm 0.11	1.17	7.3
4.5	109.0 \pm 12.3	2.04	0.23 \pm 0.09	1.40	4.3
5.0	166.0 \pm 5.9	2.22	0.20 \pm 0.05	1.60	3.0

much more variable, the probable error sometimes equalling the mean value. This is to be expected on account of the very small

amounts involved. In any case, these random errors do not affect certain very large differences involved. The smoothness of the curves and the close check of the curves for various media give evidence of the validity of the general relationships deduced.

TABLE 4
Growth and carbon dioxide yield in aerated peptone water

AGE	BACTERIA, MILLIONS PER CUBIC CENTIMETER		CO ₂ YIELD		
	Actual	Log	Increment	Cumulative total	Rate per cell per hour during previous interval mgm. $\times 10^{-11}$
hours			mgm. per 100 cc.	mgm. per 100 cc.	
0	15.9 \pm 2.2	1.20			
1	14.5 \pm 2.1	1.16	0.54 \pm 0.24	0.54	37
2	22.9 \pm 3.5	1.36	2.16 \pm 0.34	2.70	123
3	85.3 \pm 13.8	1.93	3.32 \pm 0.15	6.02	73
4	236.0 \pm 32.8	2.37	7.10 \pm 0.64	13.12	50
5	655.0 \pm 80.3	2.82	9.13 \pm 1.14	22.25	22

TABLE 5
Growth and carbon dioxide yield in aerated glucose-peptone water

AGE	BACTERIA, MILLIONS PER CUBIC CENTIMETER		CO ₂ YIELD		
	Actual	Log	Increment	Cumulative total	Rate per cell per hour during previous interval mgm. $\times 10^{-11}$
hours			mgm. per 100 cc.	mgm. per 100 cc.	
0	17.3 \pm 2.6	1.24			
1	17.1 \pm 2.4	1.23	0.72 \pm 0.34	0.72	43
2	44.5 \pm 9.6	1.65	3.29 \pm 0.54	4.01	117
3	172.0 \pm 22.7	2.24	5.76 \pm 0.45	9.77	63
4	585.0 \pm 40.7	2.77	11.81 \pm 0.44	21.58	35
5	861.0 \pm 101.8	2.94	11.07 \pm 0.28	32.65	16

GROWTH CURVES

The results, so far as bacterial growth and total cumulative yield of ammonia and carbon dioxide are concerned (columns 2 and 5 in the tables), are presented graphically in figures 1 and 2. It will be noted that the initial numbers of bacteria varied from

10.4 to 17.3 in millions per cubic centimeter; that these numbers generally dropped slightly during the first hour and then rose slightly during the next hour. In the three series with peptone

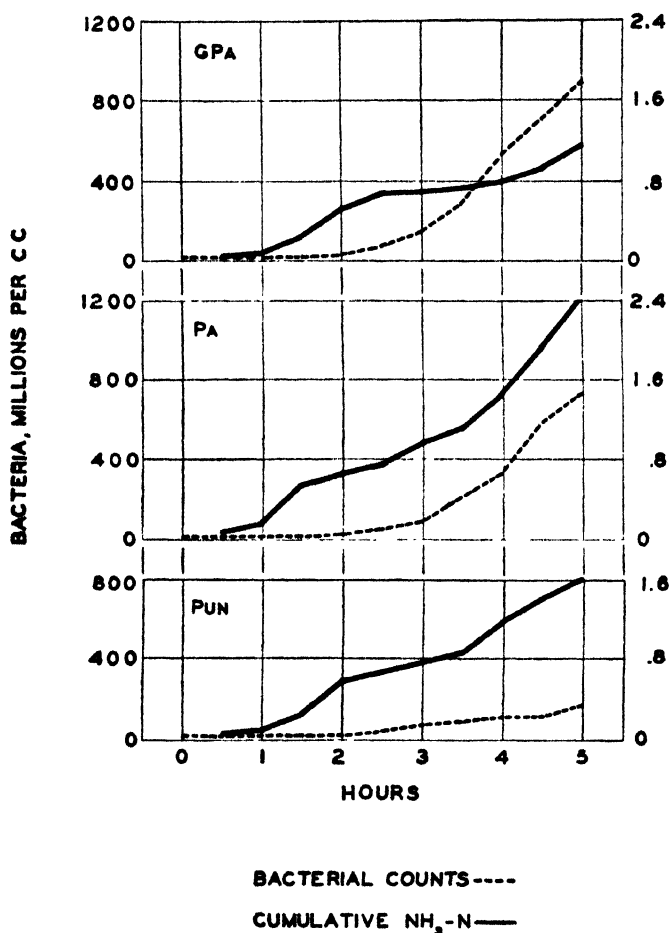


FIG. 1. BACTERIAL COUNTS AND CUMULATIVE YIELD OF AMMONIA NITROGEN
GPA = Glucose-Peptide Medium, aerated; PA = Peptone Water Medium, aerated; PUN = Peptone Water Medium, unaerated.

medium the lag period extended further, the two-hour count being less than 200 per cent of the initial count. In the glucose

peptone medium on the other hand the period of logarithmic increase began after 1.5 hours (see table 2). From the second to the third hour the population increased four-fold. This approximates most closely the true phase of logarithmic increase. After the third hour in the unaerated peptone medium and after the fourth hour in the other media, the rate of increase slackened.

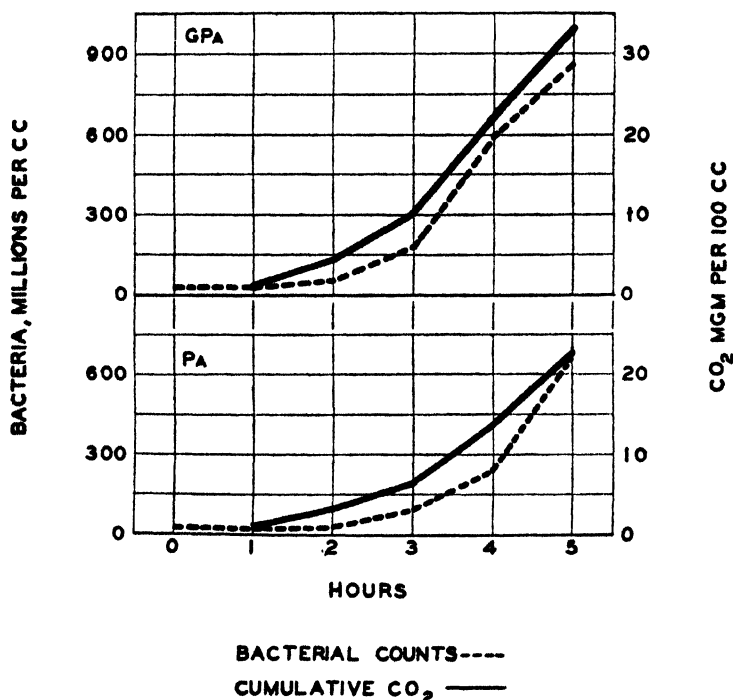


FIG. 2. BACTERIAL COUNTS AND CUMULATIVE YIELD OF CARBON DIOXIDE

GPA = Glucose-Peptide Medium, aerated; PA = Peptone Water Medium, aerated.

The final counts varied from 655 to 890 million per cubic centimeter, except in the case of the unaerated peptone medium where the number reached only 166 million. The effect of aeration in a peptone medium was, then, to prolong the period of logarithmic growth and to increase the final count. The effect of adding glucose to an aerated peptone medium was to initiate logarithmic

growth more promptly, without greatly affecting the ultimate population. The relative duration of the lag and logarithmic

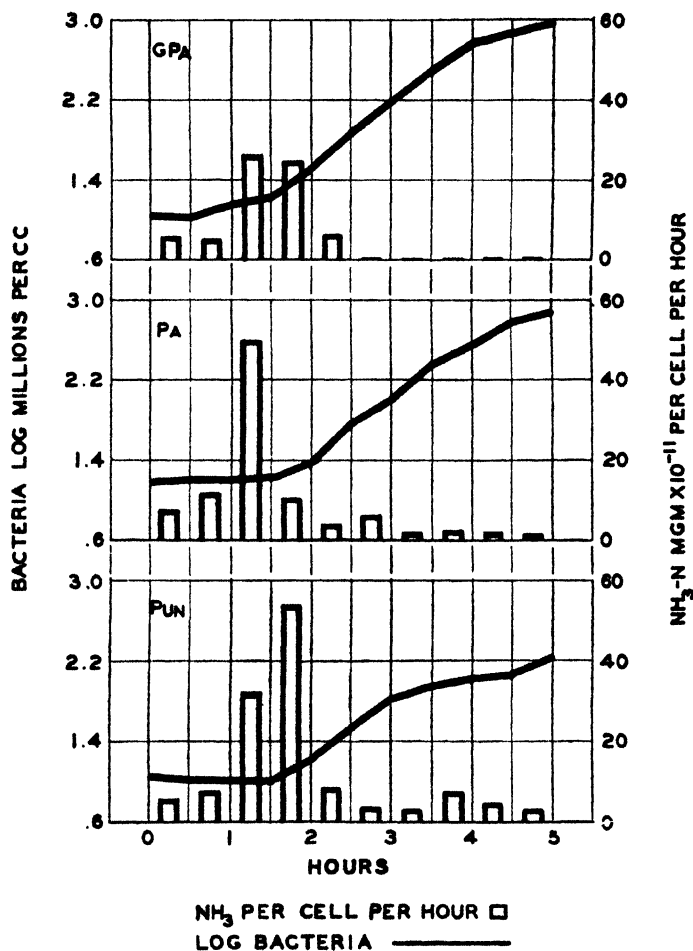


FIG. 3. LOGS OF BACTERIAL COUNTS AND YIELD OF AMMONIA NITROGEN COMPUTED PER CELL PER HOUR

GPA = Glucose-Peptone Medium, aerated; PA = Peptone Water Medium, aerated; PUN = Peptone Water Medium, unaerated.

periods and the characteristics and velocities of growth in the several experimental series may easily be compared in the logarithmic population curves of figures 3 and 4.

AMMONIA YIELD

It will be noted from figure 1 that the curve for the total ammonia nitrogen added to the medium by the bacteria goes up very sharply during the second hour—that is, just prior to the initiation of rapid logarithmic growth. The curve then flattens out some-

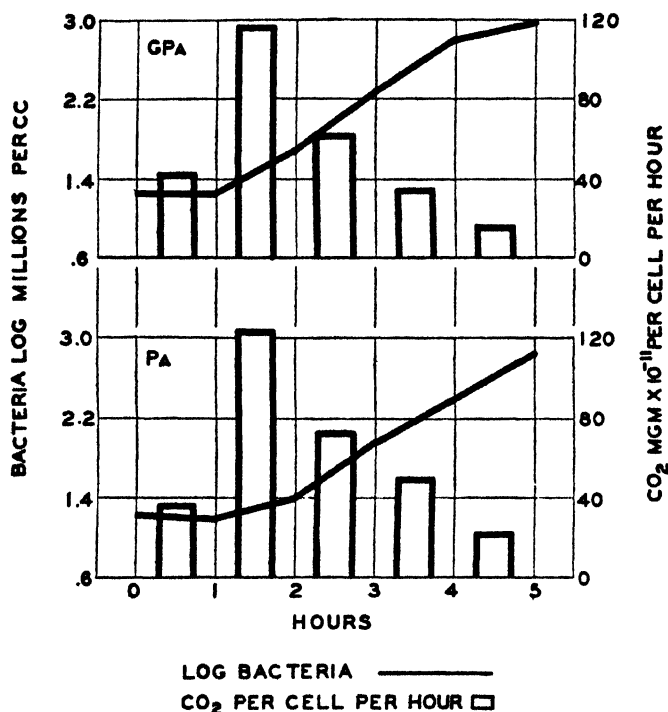


FIG. 4. LOGS OF BACTERIAL COUNTS AND YIELD OF CARBON DIOXIDE COMPUTED PER CELL PER HOUR

GPA = Glucose-Peptone Medium, aerated; PA = Peptone Water Medium, aerated.

what and later rises as the total bacterial population rises. It is obvious, however, even from these linear graphs, that something has happened to change the rate of bacterial metabolism during the late lag phase.

In figure 3 the course of the bacterial population has been plotted on a logarithmic basis, to bring out more clearly the

points of inflexion and relative rates of increase; and the ammonia yield has been indicated as a rate per cell per hour. The ammonia yield is greatest in the unaerated peptone medium, averaging for the whole five hours, $12.9 \text{ mgm.} \times 10^{-11}$ per cell per hour. It is somewhat lower in the aerated peptone, averaging $9.8 \text{ mgm.} \times 10^{-11}$ per cell per hour and is still lower in the aerated glucose-peptone medium, the value falling here to $7.0 \text{ mgm.} \times 10^{-11}$ per cell per hour. This last drop is, of course, consistent with the familiar sparing action of carbohydrates.

In all media, however, the burst of adolescent activity at the close of the lag period and the beginning of the period of logarithmic increase is very clearly shown. During the first hour the values for all three cultivation conditions were between 5.1 and 11.8. During the second hour they rose to averages of over 25 for the aerated glucose-peptone medium, over 30 for the aerated peptone medium and over 40 for the unaerated peptone medium. The sharpest peak occurred during the third half-hour in the aerated peptone and during the fourth half hour in the unaerated peptone medium; while it was divided equally between the third and fourth half hours in the aerated glucose-peptone medium. In the aerated peptone the peak came at the end of the lag period, in the unaerated peptone at the end of lag and the beginning of the logarithmic phases, in the aerated glucose-peptone medium at the beginning of the logarithmic period.

After the second hour (during the course of the logarithmic period) the values fell sharply again to rates between 0.3 and 8.4 $\text{mgm.} \times 10^{-11}$ per cell per hour.

This subsequent decline does not, of course, necessarily imply a correspondingly reduced production of ammonia per cell per hour. It is quite possible that, as the bacteria undergo more rapid multiplication, they may utilize in the synthesis of new protoplasm some of the already formed ammonia-nitrogen in the medium at a rate sufficient to mask the further destruction of peptone. The initial burst of activity is, of course, obvious; and we know from our earlier studies that a very low balance of ammonia yield is manifest in the stable phase of the population cycle. Just how fast the curve of actual ammonia production falls during the logarithmic growth phase is, however, uncertain.

CARBON DIOXIDE

Corresponding data with regard to carbon dioxide yields and rates are presented in figures 2 and 4, except that no tests were made without aeration, since with our technique the carbon dioxide could not be determined with accuracy without the use of an aeration train.

The average carbon dioxide yield for the whole five hours was 55 mgm. $\times 10^{-11}$ per cell per hour for the glucose-peptone medium and 61 mgm. for the plain peptone water. The average hourly rates and the ratios of the products are summarized in table 6. The rates for hourly ammonia do not correspond entirely to the averages of the

TABLE 6

Average hourly yield of ammonia and carbon dioxide in peptone and glucose peptone media (aerated)

HOURS	NH ₃ N PER CELL PER HOUR MG. $\times 10^{-11}$		CO ₂ PER CELL PER HOUR MG. $\times 10^{-11}$		RATIO CO ₂ /NH ₃ N			
	Peptone	Glucose-peptone	Peptone	Glucose-peptone	Peptone	Glucose-peptone	Peptone	Glucose-peptone
0-1	10.4	5.0	37	43	3.6	8.6	1.1	2.7
1-2	26.7	21.3	123	117	4.6	5.5	1.5	1.7
2-3	6.2	2.3	73	63	11.8	27.4	3.8	8.7
3-4	2.6	0.3	50	35	19.2	116.7	6.1	37.2
4-5	1.9	0.5	22	16	11.6	32.0	3.7	10.2

half-hourly figures in tables 1 and 2 since they have been here computed (for comparability with carbon dioxide) by applying the Buchanan formula to hourly increments and periods. In spite of the lower rate of ammonia production in the presence of glucose, the carbon dioxide yield is not only no greater but is actually less when sugar is present. During each of the last four hours the rate of carbon dioxide yield per cell was actually lower in the glucose-peptone medium than in peptone water. The average of the five periods is 61.0 mgm. $\times 10^{-11}$ per cell per hour in peptone water as compared with 54.8 mgm. in the glucose-peptone. In the study of Walker and Winslow (1932) the rate of carbon dioxide production was approximately the same in peptone and in lactose-

peptone media, likewise indicating failure of the cell to show increased CO_2 output in the presence of a fermentable carbohydrate.

As in our earlier work, the molar ratio of CO_2 yield to $\text{NH}_3\text{--N}$ yield approximates one in the lag period and rises to much higher values in the logarithmic phase. This phenomenon, like others we have noted, may possibly be related to withdrawal of ammonia nitrogen from the medium by the rapidly-growing cells.

Figure 4 gives graphic emphasis to the fact that the bacteria during the first hour (lag period) produce carbon dioxide at a rate of 37–43 mgm. $\times 10^{-11}$ per cell per hour; while during the second hour the rate rises sharply to 117 to 123 mgm. $\times 10^{-11}$; and thereafter the rate of yield falls steadily to 16 to 22 mgm. $\times 10^{-11}$ for the fifth hour.

DISCUSSION OF GENERAL PHENOMENA

These results confirm those obtained by Walker and Winslow (1932) but bring out the wider differences which take place and which were masked by the longer time intervals first studied. The new data show very sharply the burst of adolescent energy which is associated with the last half hour of the lag period and the first half hour of the phase of logarithmic increase.

This phenomenon is clearly related to that described by Sherman and Albus (1923) in their paper on "Physiological Youth of Bacteria." Sherman and Albus found that young cultures of bacteria were more sensitive to the action of distilled water at 2°C ., to 2 per cent NaCl, to a temperature of 50 to 53°C ., and to 0.5 per cent phenol—and were less responsive to acid agglutination—than were older cultures. Their "young" cultures, however, were three and one-half to four and one-half hours old and their "old" cultures twenty-one hours to twelve days old. They were, therefore, probably comparing the logarithmic period with a much later stable phase, rather than the lag period with the logarithmic phase.

In a second contribution, however, Sherman and Albus (1924) showed that this state of high susceptibility was manifest after one and one-half hours, or during the latter part of the lag period. Thus, it does represent a change initiated before the period of

logarithmic increase but one which apparently continues throughout that period.

The work of Clark and Ruehl (1919) and of Henrici (1928) presents somewhat similar phenomena. Clark and Ruehl found that of thirty-seven different species of bacteria whose morphology was studied at various phases of the growth cycle all but the diphtheria bacillus showed very much larger cells in four to nine-hour cultures than in twenty to twenty-four-hour cultures. In the case of the diphtheria bacillus the cells in young cultures were distinctly smaller than in old cultures. In general, the largest cells were observed between four and six hours at the height of logarithmic increase but with *Esch. coli*, it is interesting to note, the height of the curve was reached at 2 hours. Henrici (1928) confirmed these results and found that with *Esch. coli* and *B. megatherium* young cultures showed longer and more slender cells than old cultures. In both instances the largest cells were observed during the course of the period of logarithmic increase.

The observations of Bayne-Jones and Rhees (1929) on heat production by young cells are closely in line with our CO_2 and NH_3 rate data. These investigators measured, in an apparatus specially designed for bacterial calorimetry, the heat production per cell in a unit interval of time and recorded a very sharp peak of heat production during the second hour of incubation. Their results check very perfectly with our own and point with equal clearness to a period of marked metabolic activity at the beginning of the phase of logarithmic increase and not at its height. Criticism of the methods by which Bayne-Jones and Rhees computed their rates per cell per hour does not affect the general results, as shown by the re-calculations of Rahn (1930). An important theoretical analysis of the energetics of the processes involved in such phenomena has recently been offered by Wetzel (1932) using Bayne-Jones' data and applying a formula which he believes applicable to growth and metabolism throughout the biological field.

Burk and Lineweaver (1930) in a study of the "respiration rate" of azotobacter found that the ratio of growth increase to increase in oxygen consumption rose as the cultures aged, a phenomenon

which obviously involves a higher metabolic rate in the earlier hours. Cutler and Crump (1929) give data on carbon dioxide production in soils by pure cultures of soil organisms, which show a high rate of production during young active growth, and Meiklejohn (1930) presents results on ammonia production by soil bacteria which appear to illustrate the same phenomenon. Boyd and Reed (1931) describe a sharp fall in oxidation-reduction potential in cultures of *Esch. coli* at the onset of the logarithmic growth period, which slackened somewhat in the later hours of this phase, when no sugar was present. Eaton (1931) gives curves which show quite clearly that the "respiration rate" of a staphylococcus culture goes up faster than the growth curve at first and more slowly at a later period. The author states that as the culture ages, the rate of respiration gradually decreases. Gerard and Falk (1931) noted in aqueous and glucose suspensions of *Sarcina lutea* an initial high rate of oxygen consumption, which declined during later hours, although the organisms were undergoing active growth.

Martin (1932) in Bayne-Jones' laboratory has demonstrated a clearly defined period of intense physiological activity per cell following the close of the lag period as measured by the oxygen consumption of *Esch. coli* and by the surface area of the average organism. Furthermore, he compares our earlier results and those of Bayne-Jones and Rhees from a quantitative standpoint and shows that they are closely related with each other and with his own.

In connection with this work, Martin presented data on the size of the cells of *Esch. coli* at one-half hour intervals. The largest cells were noted at 1, 1½ or 2 hours after inoculation, which points fall early in his logarithmic growth periods.

Finally, Bayne-Jones and Adolph (1932) and Bayne-Jones and Sandholzer (1933) have confirmed and extended the findings of Henrici and Martin on the cell size of *Esch. coli* at various phases of the life cycle. We shall refer again to these results in a later paragraph.

From the foregoing discussion it is apparent that a number of workers have presented evidence in terms of several metabolic

indices—oxygen consumption, heat production, carbon dioxide production, ammonia yield, and the like—reflecting a period of high activity per cell at the close of the lag and the beginning of the logarithmic growth phases. The agreement between these various researches is on the whole most striking.

INTERPRETATION

All this material seemed to us, at first, as it has seemed to others, to demonstrate beyond question the existence of a period

TABLE 7

Comparison of various characteristics at different phases of the life cycle

	HOURS			
	0-1	1-2	2-3	3-4
Oxygen consumption* per cell per hour, mgm. $\times 10^{-11}$	46.7	72.5	49.5	26.6
Heat production,† gram calories per cell per hour, $\times 10^{-11}$	87.5	247.7	92.6	25.7
Carbon dioxide production‡ per cell per hour, mgm. $\times 10^{-11}$	36.5	123.0	73.0	50.0
Ammonia nitrogen‡ per cell per hour, mgm. $\times 10^{-11}$	10.4	26.7	6.2	2.6
Average volume of cells in cubic micra§.....	1.9	3.1	1.0	0.8

* Computed from Martin (1932), table 1, p. 697.

† Data of Bayne-Jones and Rhees (1929). Recalculated from table 2, p. 131, using Buchanan formula.

‡ Data from present paper, table 6.

§ Computed from data used by Bayne-Jones and Sandholzer (1933) in preparing upper curve of table 1, p. 284. Courteously furnished by Dr. Bayne-Jones.

of physiological youth at the end of the lag phase of the culture cycle. It then occurred to us, however, that the variations in cell-size, recorded by Clark and Ruehl, Henrici, Martin and Bayne-Jones might have a more direct relation to metabolic activity than had been pointed out. From the data of Bayne-Jones and Rhees and Martin and from our own, we therefore made the suggestive computations summarized in tables 7 and 8.

It will be noted by reference to table 8 that the difference in cell volume between the second and the fourth hour is substan-

tially greater than the differences in oxygen consumption and in production of carbon dioxide. Thus, the higher metabolic activity per cell, as measured by these two factors, could be more than accounted for by the greater volume of the individual cells. The greater excess of ammonia production during the second hour might be due in considerable part to re-utilization during later

TABLE 8

Comparison of data in table 7 computed as ratios of values for fourth hour

	HOURS			
	0-1	1-2	2-3	3-4
Oxygen consumption.....	1.8	2.7	1.9	1.0
Heat production.....	3.4	9.6	3.6	1.0
Carbon dioxide production.....	0.7	2.5	1.5	1.0
Ammonia nitrogen production.....	4.0	10.3	2.4	1.0
Cell volume	2.4	3.9	1.3	1.0

TABLE 9

Heat production per cubic micron of bacterial substance

(Gram calories $\times 10^{-11}$ per hour)

	HOURS			
	0-1	1-2	2-3	3-4
Observed*	46	80	93	32
Computed from oxygen consumption† ..	74	70	148	100
Computed from carbon dioxide production‡	42	87	159	136

* Recalculated from data of Bayne-Jones and Rhees (1929), table 2, p. 131.

† Computed from data of Martin (1932), table 1, p. 697.

‡ Computed from our own data. Cell volumes used were in all cases those provided by Bayne-Jones (see footnote to Table 7).

phases of ammonia nitrogen liberated from easily disrupted peptone fractions during the first hours. Only the excess of heat production remains abnormally high during the first hours.

Following the example of Martin in attempting to discover relationship between the O_2 , CO_2 and heat data, we have computed in table 9 the heat production per hour per cubic micron of bacterial substance (from Bayne-Jones and Rhees) and compared

it with the heat production which might be expected to correspond to the oxygen consumed (Martin) and to the carbon dioxide produced (our own data). Despite the many assumptions involved it is interesting to note that the values are of the same general order of magnitude, with somewhat better agreement between heat predicted by O_2 and by CO_2 than between either of these and heat observed.

We recognize that such comparisons can only be rough and suggestive, since O_2 , CO_2 , and calories were each obtained by separate workers, with no absolute consistency among all three as to strain of organisms, medium composition, temperature control, degree of aeration, cell-counting methods, and the like. In general, correspondence among these factors in the work of Martin and Bayne-Jones and Rhees was close, but the specific figures used in table 9 from their work are based on single experiments. Cultivation conditions in our CO_2 experiments differed somewhat from the others and the figures used are averaged from eight cultures. Moreover, rates per cubic micron for all the data have been made dependent on bacterial dimensions supplied only by Bayne-Jones, and involve the assumption that all cells of *Esch. coli* were of the same average size during each hour. Because of all the inescapable variables involved, we have here preferred not to complicate calculations by the assumption of a respiratory quotient or by too severe refinement of a factor representing the caloric equivalent of one mgm. of O_2 or CO_2 . The figure chosen (3 calories per milligram O_2 ; 2.2 calories per milligram CO_2) was based on values commonly given in handbooks for the combustion of carbon; it also agrees approximately with values calculated long ago by Rubner for oxidation of proteins, when allowances were made for heat of solution and heat of combination by the carbonic acid.

As a further suggestive cross-check, we have calculated on the basis of average hourly CO_2 rates per cubic micron, and average hourly O_2 rates per cubic micron, (computed from Martin's data), the approximate respiratory quotients ($R.Q. = \text{vol. } CO_2 / \text{vol. } O_2$) for each hour. The figures obtained were respectively, for successive hours after inoculation, 0.57, 1.23, 1.07 and 1.37. We place no emphasis on these as absolute values and attach no signifi-

cance to the variations; the astonishing thing is that they come out anywhere near the well-known values published for various kinds of resting or growing aerobic or anaerobic bacteria, whose published R. Q.'s have usually lain between 0.7 and 1.3. Values above 0.82 in our sugar-free media might readily be attributable to differences between Martin and ourselves in the cell-counting technic on which the rates were originally based, or to differences between average hourly cell sizes in our cultures and in his, or to increased aeration leading to increased O_2 consumption by our cultures; or apart from differences in technic, slightly high R.Q.'s might be due to occurrence of some partially anaerobic activities by the cultures.

It will be noted that when computations are made, as in table 9, on a basis of cell volume, it is the third rather than the second hour which shows maximum metabolic activity.

SUMMARY

1. This study indicates that when *Esch. coli* is grown in a peptone-water medium, with or without continuous aeration with CO_2 -free, NH_3 -free air, there is a burst of metabolic activity (estimated on a cell-per-hour basis) at the close of the lag period and before the phase of logarithmic increase has much more than begun. The NH_3 -N yielded up to the medium per cell per hour is at this time (third or fourth half hour) five times what it is during the first hour and more than ten times what it is toward the end of the logarithmic phase, i.e., after the third or fourth hour. The yield of CO_2 in an aerated medium shows a similar maximum during the second hour of three times the first-hour value and over five times the fifth-hour value.

2. The effect upon growth and metabolism of adding glucose to the peptone water is surprisingly slight in a medium continuously aerated with CO_2 -free air. The logarithmic phase is initiated somewhat more promptly but the ultimate level of growth is much the same. There is a definite sparing action shown by a lessened yield of NH_3 -N (an average reduction of about 33 per cent) but the yield of CO_2 is also reduced about 10 per cent. In a medium continuously aerated with nitrogen instead of air, so that defi-

nately anaerobic conditions are produced, the effect of glucose is very different, as we have shown recently (Walker, Winslow and Mooney (1934)).

This study adds further evidence to the demonstration of a period of marked adolescent activity beginning in the late lag period of a bacterial culture cycle and including an increase in the size of the individual cell (Clark and Ruehl, Henrici, Martin, Bayne-Jones and Adolph), increased susceptibility to antiseptic agents (Sherman and Albus), decreased oxidation-reduction potential (Boyd and Reed), increased oxygen consumption (Martin) and increased heat production (Bayne-Jones and Rhees), as well as an increased yield of carbon dioxide and ammonia nitrogen.

4. It appears, however, that a considerable part of this apparent increase in physiological activity per cell per hour, as recorded by Bayne-Jones and Rhees, by Martin, and by ourselves, can be directly accounted for by the greater size of the bacterial cells at this period. If computed on the basis of unit volumes of bacterial substance, the differences in oxygen consumption and carbon dioxide production between the second and fourth hours would disappear and the third hour would be the period of highest metabolic activity.

5. The phenomenon of physiological youth, if we define this phenomenon as a rapid increase in cell size and in metabolic activity at the close of the lag phase and in the early logarithmic phase seems fully substantiated; but the ensuing fall in the middle of the phase of logarithmic increase and towards its end appears to be demonstrated only for cell size and heat formation. The apparent decrease in oxygen consumption and CO_2 production per cell during the course of the logarithmic phase seems primarily to be a function of the decreasing volume of bacterial substance or of surface area per cell; and even the decrease in rate of ammonia yield may perhaps be explained by decreasing cell size plus utilization of previously liberated ammonia.

6. It should be clearly understood, however, that while allowance for variations in cell-size may wipe out differences between the early lag period and the late logarithmic period on the one hand and the late lag and early logarithmic period on the other, it cannot account for the high metabolic activity of all these early

phases as compared with the stable period which follows the logarithmic phase. The metabolic activity per cell during the second hour is two to four times that of the first hour and two to ten times that of the fifth hour. This difference may perhaps be largely accounted for by cell size since Bayne-Jones and Adolph (1932) show that at fifty-five to sixty-five minutes of culture age the cell volume of *Esch. coli* may be ten times what it is at four hours. Variations in cell size cannot, however, explain the fact that during the later phase of stable population (twenty-three to twenty-eight hours) the production of ammonia per cell is only one one-hundredth and that of carbon dioxide only one-fiftieth what it is at its maximum. (Walker and Winslow, 1932.) The measurements of Henrici, Martin, and Bayne-Jones and Adolph all agree in showing that the colon bacillus has a size of about $1.5 \text{ to } 2.0 \times 0.6 \text{ to } 0.7$ micra in the early lag and late logarithmic phase as compared with a size of $4.0 \text{ to } 5.0 \times 0.9 \text{ to } 1.1$ micra in the early logarithmic phase; and Henrici carried his cultures on for 96 hours with no reduction below the 1.5×0.6 figure. Thus, the cells at their maximum are not over ten times as large as at their minimum; so that the fifty fold to one hundred fold excess in yield of CO_2 or NH_3 per cell during adolescence as compared with the stable period must involve a real difference in metabolic activity.

7. The physiological constants which may be derived from these various studies seem of some interest. It appears from approximate calculations that one cubic micron of bacterial substance (*Esch. coli*) during the first four hours after inoculation, may consume 20 to 70 mgm. $\times 10^{-11}$ of carbon dioxide, and may release 3 to 7 mgm. $\times 10^{-11}$ of ammonia nitrogen per hour, liberating in the process 30 to 90 calories of heat energy.

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THE SPECIFIC INFLUENCE OF ACIDITY ON THE MECHANISM OF NITROGEN FIXATION BY AZOTOBACTER

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Few critical data exist concerning the catalytic mechanism of biochemical nitrogen fixation taking place at ordinary temperatures and pressures. Study of the mechanism is complicated by the fact that the extent and rate of fixation are normally limited by the extent and rate of growth of the microörganism carrying out the process. The nitrogen fixed is ordinarily built up into the cell bodies, chiefly as protein. It is not given off to the circumambient medium except under unusual and probably irrelevant conditions, the yields being in any case small. So far as has been investigated the elaborated nitrogen compounds, occurring in the cell bodies or excreted, are of the same chemical nature whether the organisms obtain their nitrogen for growth from free nitrogen gas, N_2 , or from simple fixed nitrogen compounds such as urea, ammonia, nitrate or nitrite. Any specific intermediate compounds formed in the initial fixation process whereby N_2 is converted into simple fixed nitrogen compounds suitable for use in growth presumably occur in very small concentrations. Because of this fact, the mechanism must be investigated chiefly by means of kinetic studies wherein critical differentiation can be made between the velocities of growth in free and fixed nitrogen. As a step in this direction Burk and Lineweaver (1931) have already found that the growth velocity of the bacterium, *Azotobacter*, can be limited or prevented in free nitrogen at certain low concentrations of calcium (or strontium) which permit normal growth in fixed nitrogen, and that consequently calcium is essential to the fixation process itself.

In the present paper an attempt has been made to determine whether the hydrogen-ion concentration of the growth medium exerts a measurable, specific influence upon the mechanism of fixation. Gainey (1923), Fred and Davenport (1918), and Krishna (1928), using nitrogen gas as a source of nitrogen, have established that growth of *Azotobacter* in either synthetic or soil medium is sharply limited to a pH of 5.9 to 6.0 or greater. They did not, however, carry out corresponding growth measurements with fixed nitrogen above and below this pH value. In their experiments growth was measured after a period of several days. This made it difficult to maintain constant conditions of pH and nutrient concentration, and to obtain exact comparative studies. In the present investigation, growth has been measured as a function of pH and nitrogen source under otherwise approximately optimum conditions. The experiments have lasted for only six to ten hours, during which time the pH values were easily kept constant to within 0.05 unit by appropriate phosphate buffering. Growth has been logarithmic at any given pH, yielding a first order velocity constant, g , as in the formula " $g = 2.3 \text{ d log } (a + y)/dt$," where a is the initial growth and y is the growth increase after t , the time in hours. g is evaluated by plotting the logarithm of growth with respect to time (see fig. 2). It is an important and fundamental criterion of growth since it represents at all times the velocity of growth at unit concentration of cells, as distinguished from the observed velocity, which necessarily varies with the cell concentration and therefore with the time or duration. With respect to time, g is the differential, rather than the integral measure of growth.

METHODS

The Warburg manometric technique employed to measure the rate of oxygen consumption has been described in detail elsewhere (Burk, 1930). The cultures studied in the Warburg vessels were first grown in an air thermostat at 28°C. and at pH 6.8, in sterilized, aerated, 500 cc. gas wash bottles containing 50 to 100 cc. of nutrient medium. The medium consisted of 1 per cent glucose dissolved in the clear liquid remaining after the following

mixture had been thoroughly shaken, allowed to stand, and settle: 0.8 gram K_2HPO_4 , 0.2 gram KH_2PO_4 , 0.2 gram $NaCl$, 0.2 gram $MgSO_4 \cdot 7H_2O$, 0.1 gram $CaSO_4 \cdot 2H_2O$, 0.01 gram $Fe_2(SO_4)_3 \cdot 9H_2O$, 1000 grams H_2O . Young active cultures of *A. vinelandii*, or *A. chroococcum*, twelve to forty-eight hours old and containing 5 to 50 million cells per cubic centimeter, were employed. During the growth measurements in the Warburg vessels they were maintained at $31 \pm 0.01^\circ C.$, with air in the gas phase, unless it were desired to prevent all growth, in which case 21 per cent O_2 in H_2 was used. When fixed nitrogen was added to the liquid culture medium a concentration of 0.0072 N (100 p.p.m.) nitrate, ammonia or urea was used. This concentration is optimum and prevents all fixation of nitrogen, even when nitrogen gas is present as in air, and makes growth dependent entirely upon fixed nitrogen supply.

Growth was measured chiefly by increase in rate of oxygen consumption with time, which is accompanied by a proportional increase in the turbidity, dry weight, number of organisms and cell nitrogen, the latter whether derived from free or fixed nitrogen. As shown in figure 2 the manometers could be read frequently and the velocity constant of growth determined during the course of several hours. Turbidimetric measurements of growth by means of a Bausch and Lomb nephelometer were also made, chiefly at the lower pH values. Initial, final, and often intermediate, pH values were determined colorimetrically with a relative accuracy of ± 0.05 unit, with the several La Motte indicators and standards. The varied pH values of the cultures were adjusted with approximately N/20 HCl or N/20 $NaOH$, or, where somewhat increased buffer capacity was desired, with as much as one additional gram per liter of K_2HPO_4 or KH_2PO_4 , outside indicators being employed as a general rule.

The pH functions

Respiration. Figure 1 presents respiration as a reversible function of pH over the range 5 to 8.5. A reversible function is one which involves no permanent injury or inactivation. Different experimental runs are indicated by different point designa-

tions (+, o, x, ., etc.). The values were observed in cultures where growth was permitted by the presence of free or added fixed nitrogen, and also where growth was prevented by maintaining the organisms in a mixture of 21 per cent O_2 in 79 per cent of some inert gas such as H_2 , or He, the total pressure being one atmosphere as in the case of the air-grown cultures. Where growth was excluded the respiration rate at any given pH re-

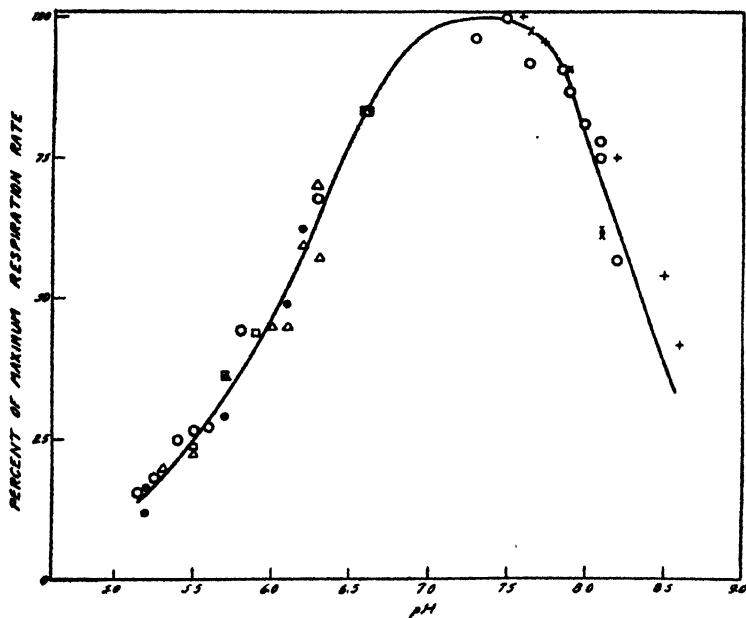


FIG. 1. RESPIRATION AS A FUNCTION OF pH

maintained practically constant with respect to time for twelve hours or more, except at the extreme ends of the function, pH 5.6 or less and pH 8.3 or more. Here irreversible inactivation took place (Burk, Horner and Lineweaver, 1932), increasing in rate and extent as the actual function limits were approached. The reversible value was consequently obtained by extrapolating back to $t = 0$. This procedure was also employed where growth, in nitrogen, took place causing the observed respiration rate per vessel to increase with time. The curve in figure 1 is, by virtue

of this more refined procedure, and because of more extensive measurements, over a wide range more accurate than a similar one given previously (Burk, Horner and Lineweaver, 1932). It is seen that the respiration rate per unit of dry matter attains its optimum value at about 7.2 to 7.5, and falls to 5 per cent or less of the optimum value in a reversible manner at about pH 4.5 and

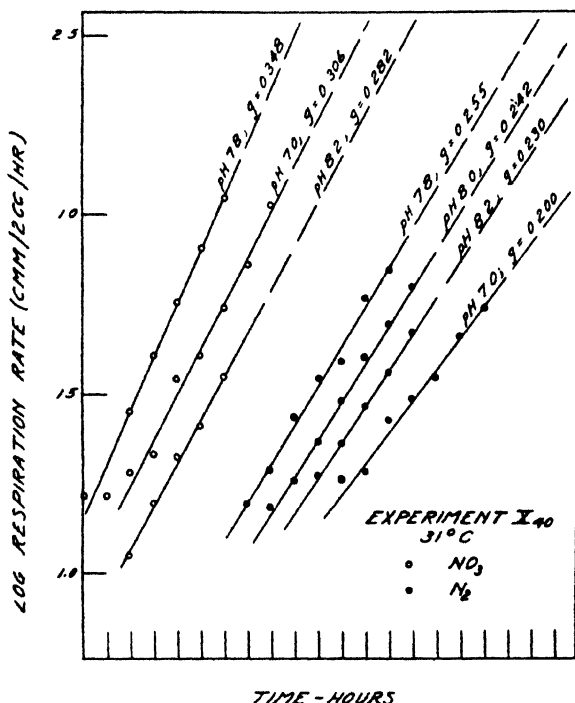


FIG. 2. ILLUSTRATIVE DATA SHOWING METHOD OF OBTAINING g , THE FIRST ORDER VELOCITY CONSTANT OF GROWTH

9.0. The fall on the alkaline side is definitely the steeper, and hence the whole curve is slightly unsymmetrical.

Growth. Figure 2 illustrates the method of obtaining the values of g , at any given pH and source of nitrogen, from the increase in respiration rate with time. All the values shown were obtained during one experimental run of seven hours and with the same initial stock culture. As a matter of convenience semi-logarith-

mic paper was employed to obtain the linear slope and g values from the respective respiration-rate values. Figure 2 illustrates only a few g values occurring in the restricted pH range 7.0 to 8.2, where g varies relatively little. The optimum values of g in both NO_3 (nitrate) and N_2 are seen to occur at about pH 7.8, as compared, in this particular instance, with pH values of 7.0 and 8.0 and 8.2.

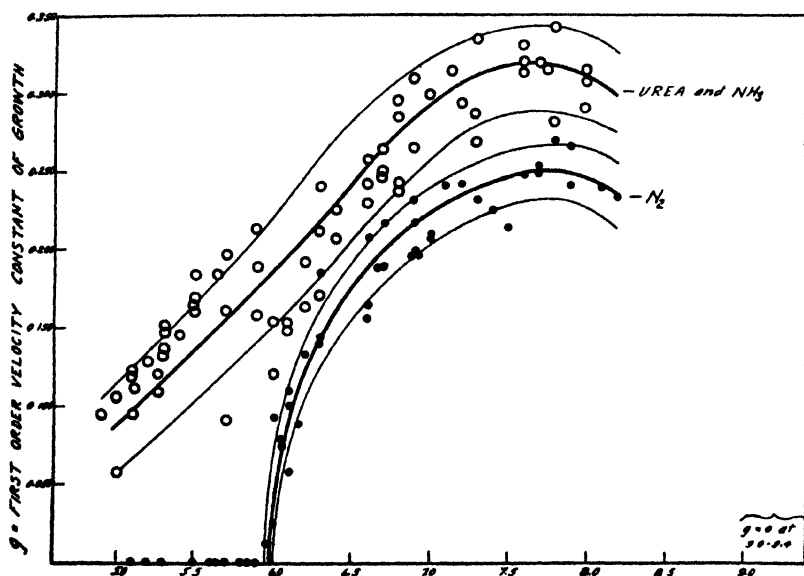


FIG. 3. GROWTH IN UREA AND AMMONIA AND NITROGEN GAS AS A FUNCTION OF pH

Figure 3 presents the g values normally obtained with free nitrogen and two forms of fixed nitrogen (urea, and NH_3 as $(\text{NH}_4)_2\text{H}_2\text{PO}_4$ or NH_4Cl) over the pH range 5 to 8. The values were obtained in a large number of experiments, those above pH 6 in the same manner as the seven illustrated in figure 2, and those below pH 6 chiefly by means of initial and final turbidimetric measurements. The latter procedure was necessary because, as mentioned, the respiration rate itself is subject to a certain irreversible inactivation below about pH 5.6, and consequently its rate of increase is no longer an accurate measure of growth. The

experiments were performed with different stock cultures during the course of a year or two. This accounts for the moderate variation of the values at any one pH, indicated by the lighter lines. The heavier lines have been drawn not only as averages of all the respective points but also with due regard to the relative values of the different experiments. A certain differentiation could be made between experiments on the basis of internal consistency and extent of replication. For this reason the heavy line in the case of urea and NH_3 has not been drawn with any marked inflection at about 5.6 to 6.0, such as a truly average curve might suggest. A closer analysis might show that this somewhat more complicated function was involved, at least in the case of urea. The velocity of growth in urea is in general slightly more rapid than in NH_3 at the higher pH values, and slightly lower at the lower ones, but the points for both have been grouped together for convenience in making comparison with the velocity in N_2 . In connection with the absolute magnitude of g , it may be mentioned that values of 0.30, 0.20, and 0.10 correspond to a doubling every $2\frac{1}{3}$, $3\frac{1}{2}$, and 7 hours, respectively, of the respiration rate, turbidity, cell number, dry weight, nitrogen fixed or utilized, etc.

Fixation of free nitrogen. Figure 3 shows that growth in free and fixed nitrogen as a function of hydrogen ion concentration can be clearly differentiated with respect to both the extreme acid limit and the manner of approaching that limit. In N_2 a sharp limit is reached at pH 5.9 to 6.0, as found by the investigators previously referred to, and the approach is concave downward. In fixed nitrogen a theoretical limit appears to be approached considerably below pH 5, possibly at 3 to 4, and the approach is asymptotic or concave upward. The optima in both free and fixed nitrogen occur at the same pH, namely 7.6 to 7.8, which lends emphasis to the differentiation between the functions in the acid range. The alkaline range greater than pH 8.2 has not been studied extensively because of certain technical difficulties. Growth appears to attain a limit at pH 9 or slightly above, undoubtedly due in great part to cessation of respiration.

Curve I, figure 4, is a replot of the N_2 -growth curve of figure 3 upon a basis of percentage of maximum activity. It represents

the combined influences of pH upon the growth process and the nitrogen fixing mechanism. Curve VIII, figure 4, presumably represents more closely than curve I the true pH fixation function. It has been obtained by dividing curve III into curve I on the assumption that curve III represents the influence of pH upon the growth process itself, with a fixed nitrogen compound requiring reduction, and gives the approximate, required correction. Curve VIII is much steeper than curve I and extends only from

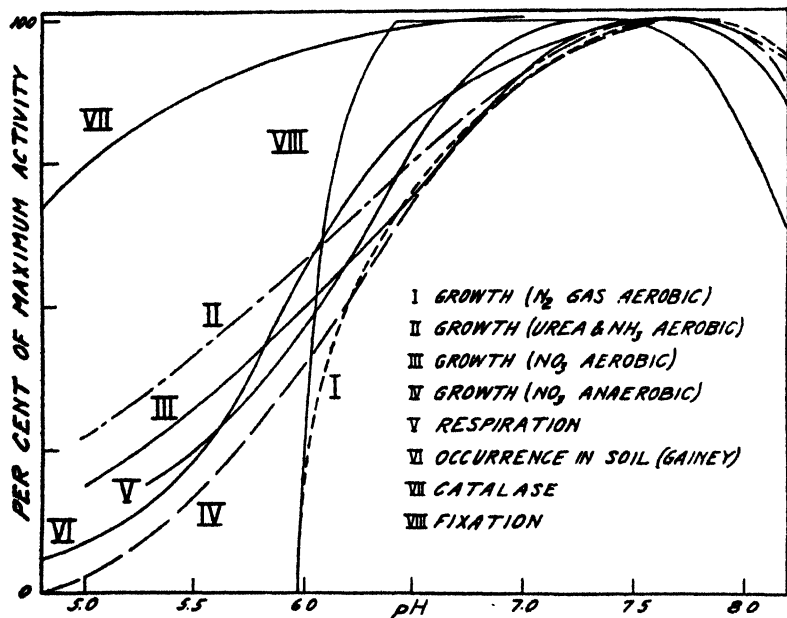


FIG. 4. VARIOUS REACTIONS OCCURRING IN AZOTOBACTER, AS A FUNCTION OF pH

pH 5.97 to 6.37 (± 0.02). A plot of the nitrogen fixation activity data so obtained against hydroxyl ion concentration instead of pH would yield a still steeper curve. A plot against hydrogen ion concentration would give a curve less steep than curve VIII but one which would still approach a zero value on the hydron concentration axis linearly or slightly concaved downward (cf. Wherry, 1928). An extended interpretation of curve VIII will be given elsewhere, in connection with phase rule considerations (Burke and Lineweaver, J. Phys. Chem., in press). Curves

I or VIII, figure 4, establish even more strikingly the distinctiveness of the N_2 -growth or fixation functions, which are contrasted further with several other functions of *Azotobacter* given by curves III, IV, V, VI and VII, respectively: aerobic growth in nitrate; anaerobic growth in nitrate; respiration; occurrence in nature;¹ and catalase activity (Burk, Horner and Line-weaver, 1932). The other functions are comparable to the growth function in urea or NH_3 (curve II) in approaching low pH limits. With the possible exception of catalase activity, they make that approach in a manner that is concave upward.

In figures 2 and 3, the g values at optimum pH are 20 to 30 per cent lower in N_2 than in fixed nitrogen. This is true chiefly (but not entirely) because the N_2 pressure of 0.78 atmosphere in air provides only about 80 per cent of the maximum velocity, which is attained to the extent of 98 per cent or more at 4 to 8 atm. (Burk and Milner, 1932, fig. 4). The latter high pressures would be very inconvenient to employ experimentally with complete satisfaction. Our most recent work indicates that the concentration of fixed nitrogen (nitrate, ammonia or urea) yielding optimum velocity is less than 0.1 p.p.m. ($10^{-4}N$), whereas 100 p.p.m. were employed, chiefly in order that the concentration should not decrease appreciably during the periods of meas-

¹ Confirmation of the finding that growth of *Azotobacter* occurs in fixed nitrogen well below pH 6.0 may be derived from curve VI, figure 4, which is a replot of some of the extensive work of Gainey (1924) and Gainey and Batchelor (1923). This curve shows the relative occurrence of *Azotobacter* in several hundred soils examined. Growth and maintenance of the organism in soils below pH 6.0 was presumably possible by virtue of fixed nitrogen compounds present therein. Fixation never took place in these soils unless they were both brought to and maintained above pH 6.0 with alkali or carbonate. This latter procedure was employed by Gainey in demonstrating the occurrence of *Azotobacter* under the more acid conditions. He did not determine its presence directly by adding fixed nitrogen and carbohydrates to the soils more acid than pH 6.0. Under these conditions other organisms might easily have developed somewhat more readily than *Azotobacter* and hindered detection of the latter. Such a circumstance is generally not significant in soils above pH 6.0 where a large amount of carbohydrate but no fixed nitrogen has been added. From the agronomic point of view, it is reasonably certain that application of nitrogenous fertilizers to soils under field conditions will often increase the absolute numbers of *Azotobacter* therein, as in the case of other microorganisms.

urement. Since the experiments in free and fixed nitrogen reported in figures 2, 3, and 4 therefore differed somewhat with respect to the relative saturation concentration employed, additional experiments were performed at low concentrations of fixed nitrogen, namely, 1 to 5 p.p.m. of urea, NH_3 and NO_3 . It was found that the ratio of growth velocities above and below pH 6.0, *e.g.*, at pH 5.25 and 6.2. were substantially those obtained at 100 p.p.m. Moreover, the last traces of fixed nitrogen at very low initial concentrations were removed equally completely at the low as well as at the high pH values, at least down to the limit of experimental analysis, namely, to 0.1 p.p.m. Additional evidence on this point of control is also provided by curve IV, figure 4. Whereas g at optimum pH and under aerobic conditions is some 25 per cent lower in free than in fixed nitrogen, the absolute magnitude of g under optimum conditions of anaerobic growth in nitrate is actually about 90 per cent lower; yet the functions in the two latter cases are qualitatively similar. In view of either of these two very different circumstances the markedly different g functions obtaining in free and fixed nitrogen in the acid range can not be ascribed in any way to the former being somewhat smaller in magnitude at the optimal pH. It is also to be remembered in this connection that slight but definite differences occur between the aerobic growth velocities in the fixed nitrogen compounds themselves, in the order urea and ammonia, nitrate, nitrite, nitrogen gas (fig. 4; and Burk and Milner, 1932, fig. 6).

Erlenmeyer technique. As further confirmation of figures 3 and 4 we have grown *Azotobacter* in free nitrogen and in several forms of fixed nitrogen in synthetic media buffered well below pH 6 in Erlenmeyer flasks or aeration bottles (Gainey, 1924, and Burk and Lineweaver, 1931), in the usual manner with which investigators in general are familiar, rather than by the Warburg technique. Such experiments with fixed nitrogen have lasted several days and very heavy growths have been obtained, *viz.*, 10 mgm. organic nitrogen per 100 cc., at a constantly maintained value of pH 5. Under the same conditions growth with free nitrogen occurred, as before, only above pH 5.9 to 6.0. As a matter of fact, in the absence of sufficient buffer, the pH will automatically fall, in the

case of organisms grown in NH_3 , to a pH of about 4.0 to 4.5, due to acidity caused by its absorption. This appears to be about the true acid limit of measurable growth, as distinguished from the previously mentioned theoretical, extrapolated value of pH 3 to 4. This measurable limit appears, further, to be caused chiefly by the complete cessation of respiration at this point. Such respiration inhibition occurs not only as a reversible function of pH but in fact chiefly because of irreversible inactivation, which is very pronounced below pH 4.8 to 5.0. As will shortly be indicated, even at pH 4 growth itself is subject to only slight irreversible inactivation.

Fixation reversibility. The important point has been established that the N_2 -growth curve presented in figure 3 is not only reversible above pH 6, but that irreversible inactivation of the nitrogen-fixing mechanism does not occur until the organisms are exposed to pH values at least below 5. A culture was divided into four fractions and these were maintained for 11 hours at pH 7.0, 5.9, 5.6, and 5.1, respectively, by appropriate addition of dilute HCl. All subcultures were brought to pH 7.0 with dilute NaOH and their growth velocities in free and fixed nitrogen measured. All subcultures in nitrate gave g values of 0.27 ± 0.02 and all those in N_2 gave 0.20 ± 0.01 . These g values were established within at least two hours after the final pH readjustments. A similar experiment with exposures at lower pH values for twenty hours indicated that g in nitrate was slightly reduced at pH 4, possibly 10 to 20 per cent for the subsequent ten hours following pH readjustment, whereas g in N_2 was reduced by at least 75 per cent during this period and did not wholly return to normal even after twenty-four hours.

Influence of pH and other factors on Michaelis constant of fixation. Brief mention should be made here of comprehensive experiments, to be detailed elsewhere in other connections, which show that the nitrogen pressure function is independent of pH over the range in which the growth velocity in free nitrogen is being markedly affected. Between pH 6.05 and 7.2 the Michaelis dissociation constant, K_m (Haldane, 1930, p. 39), does not change. This constant is the nitrogen pressure at which, at any given pH,

half the limiting velocity is reached. It remains constant at 0.22 ± 0.02 atm. In other words, any inhibition of fixation by hydrogen ion is non-competitive; the hydrogen ion does not unite directly with the identical chemical grouping which combines with nitrogen gas. Further evidence confirming this point was obtained by growing *Azotobacter* in Erlenmeyer flasks at elevated N_2 pressures, the flasks being placed in a large pressure bomb. Even with a 10-fold increase in nitrogen pressure ($N_2 = 8$ atm., $O_2 = 0.2$ atm.) no measurable growth in free nitrogen occurred below pH 6.0 over a period of ten days. Thus, fixation below pH 6.0 can not be made to take place by using high nitrogen pressures. In cultures maintained above pH 6.0 the slightly beneficial influence of the increased nitrogen pressure on the growth velocity was independent of the pH.

Calcium is the one element known so far² to be specifically required in the fixation process, being replaceable only by strontium (Burk and Lineweaver, 1931). Erlenmeyer flask technique experiments lasting several days or weeks were therefore performed with high and ordinary pressures of nitrogen and with limiting concentrations of either calcium or strontium. The limitation was obtained in two ways. Concentrations as low as one-hundredth those customarily employed were used, and also customary concentrations with varying additions of oxalate capable of precipitating the calcium or strontium. At the greatly reduced concentrations of these elements the rate and extent of fixation were greatly reduced. At sufficiently concentrated oxalate solutions (*e.g.*, 0.003 M in the case of calcium) fixation was completely inhibited. Independently of the calcium or strontium concentration, high, as compared with ordinary, nitrogen pressures caused the same fractional, small, beneficial effect on the rate of fixation. K_{mN_2} was thus not altered, even though the maximum velocity of fixation at high, saturating, nitrogen pres-

² Molybdenum (partially replaceable by vanadium) has recently been found to be specifically required in the nitrogen-fixing process, in addition to calcium. The investigations are briefly described in a general review on all our previous work on fixation: D. Burk, "Azotase and Nitrogenase in *Azotobacter*," a chapter in *Ergebnisse der Enzymforschung*, Vol. III, edited by F. F. Nord and R. Weidenhagen, Leipzig, 1934, in press.

tures decreased greatly as the concentrations of calcium or strontium became more and more limiting. This result was to be expected, of course, if either of these elements were an essential constituent of some particular enzyme system, in a conjugate nitrogen-fixing enzyme system, since the limiting concentrations would presumably lower the effective concentration of the particular enzyme, but would only under unusual circumstances affect the characteristic N_2 dissociation constant. Warburg technique experiments over the range where fixation is a more sensitive function of N_2 pressure (0 to 0.78 atm.) further confirmed the finding that $K_{mN_2} = 0.22$ atm. is independent of calcium or strontium concentration. Since oxalate did not alter K_{mN_2} , its inhibition is non-competitive. Like hydrogen ion, oxalate is a specific inhibitor of fixation and not directly involved in the chemical grouping in the system with which the nitrogen gas combines. It may be added that oxalate inhibition is reversible and may be overcome by sufficient calcium addition.

Influence of calcium and other factors on pH limit of fixation. A large number of experiments were performed, using Erlenmeyer flask technique, in which the calcium concentration (soluble and insoluble) was varied from one hundredth to one hundred times that customarily employed. The pH was varied from 5.0 to 7.0 and the nitrogen pressure from 0.8 to 8 atm. ($O_2 = 0.2$ atm.). It is to be noted here that the solubility of calcium in the presence of phosphate increases greatly between pH values of about 6.2 to 5.7 due to the conversion of dicalcium phosphate to monocalcium phosphate, the solubility of which is several hundred fold greater. Under no circumstances was any change observed, as a decrease or increase, in the pH limit of 6.0. This indicates that the catalytically active calcium is not involved as an insoluble or undissociated compound whose solubility or dissociation increases with hydrogen ion in the manner of some ordinary insoluble or undissociated base.

Similar experiments with strontium replacing calcium in the culture medium were performed with identical results. In one experiment lasting five days the amounts of nitrogen fixed at pH 7.1, 6.4, 6.1, 6.0 and several values between 5.9 and 4.6 were 134,

70, 8, 5 and 0 relative (turbidity) units. The corresponding amount of fixed nitrogen consumed with nitrate in the medium at pH 5.4 was 470 units. The values reported here were based on cultures grown in triplicate. The fixation pH limit of 6.0 was thus not affected. This finding was further confirmed with Warburg technique experiments similar to those illustrated in figure 3, strontium again replacing calcium. In one particular experiment the g values at pH 7.15, 6.30, 6.05, 5.95, 5.8, 5.7 and 5.5 were 0.22, 0.18, 0.14, 0.00, 0.00, 0.00 and 0.00, respectively. Cultures supplied with nitrate nitrogen instead of N_2 gave g values of 0.27 and 0.13 at pH 7.3 and 5.6. Here again, as in figure 3, g changed abruptly in N_2 from a moderate finite value to zero between pH 6.05 and 5.95, due to some change in the nitrogen-fixing mechanism. Most or all of the relatively much smaller decrease between pH 7.15 and 6.3 to 6.1 can be accounted for, as in the case of the calcium cultures of figure 3, by the effect of pH upon the growth process proper. All strontium cultures used in experiments reported in this paper were first grown for several transfers in a calcium-free medium containing 0.05 or 0.1 gram $SrSO_4$ per liter.

The limit of fixation at pH 6.0 has been universally observed by all investigators. Intentionally or otherwise, a great variety of experimental conditions, chemical and physical, must have been employed. An observable change in the limit is thus rendered fairly improbable. A change is conceivable, but only from intervention of some very unusual factor.

Fixation enzyme nomenclature. Azotobacter evidently possesses a highly specific chemical system of constant and characteristic properties capable of catalyzing the fixation of nitrogen gas. The essential properties of this enzyme system so far demonstrated are (1) a critical concentration of hydrogen ion greater than $10^{-6}M$ will prevent its operation; (2) calcium or strontium is required; (3) the hydrogen ion and oxalate cause non-competitive reversible inhibition; (4) the Michaelis dissociation constant, K_{Mn_2} , is 0.22 atm.; (5) K_{Mn_2} and the pH limit 6.0 are unaffected by the concentration of calcium or strontium. Also, as will shortly be shown, the system very probably consists in part of a

non-aqueous phase invariant in composition, and basic, and is affected by the oxidation-reduction potential of the medium. It is proposed here to term this specific enzyme system azotase, in accordance with accepted definition and convention (Bayliss, 1924; Cannan, 1928; Haldane, 1930; and Hitchcock, 1932). This name is derived from the substrate nitrogen gas, or azote, upon which the system acts, with deference to the name of the organism in which it occurs. The particular enzyme within the system combining directly with N_2 with a characteristic dissociation constant will be termed nitrogenase, and will be referred to as AN_2 . The calcium or strontium components of the system will be referred to as Ac_{Ca} or Ac_{Sr} , the component requiring a minimum hydroxyl ion concentration ($10^{-6}M$) as AOH^- , and the residual form of the latter component as it exists below pH 6 as AH^+ . The azotase system A thus consists of AN_2 , Ac_{Ca} , (or Ac_{Sr}), AOH^- , etc., and is comparable to the classical zymase-enzyme system, consisting of either one or more true enzymes (nitrogenase, like the phosphatases and the carboxylase); simple inorganic constituents (hydroxyl ion and calcium or strontium, like the phosphate); and very probably other constituents, as yet undiscovered (like cozymase). So far, the azotase function has been strictly endocellular, and, moreover, limited in activity by the extent of growth. Freedom from growth limitation, and eventual extracellular isolation, are being attempted.

SUMMARY

1. The rate of consumption of free nitrogen gas by *Azotobacter* decreases from a maximum at pH 7.8 to a zero limit at 6.0 (5.97 ± 0.02). The approach is perpendicular (concave downward) and reversible. Irreversible inactivation takes place only below 5.0.
2. The rate of consumption of fixed nitrogen, viz., urea, ammonia, nitrate, decreases from a maximum at pH 7.8 to a considerably more acid, measurable limit, at 4.5 or less. The approach is asymptotic (concave upward) and entirely reversible above 4.5. The rate of oxygen consumption as a function of pH is similar in type.

3. The limiting pH value 6.0 for fixation is a characteristic constant. No factors are known capable of altering this limit; in particular, calcium or strontium concentration and nitrogen pressure, three factors like pH which specifically influence fixation as distinguished from growth or respiration, fail to do so.

4. The Michaelis constant of fixation, $K_{m_{N_2}} = 0.22$ atm., or the pressure at half maximum velocity, is independent of pH, and calcium or strontium concentration. Inhibition of fixation by hydrogen ion and oxalate is non-competitive.

5. The nitrogen fixing enzyme system has been termed azotase and its known properties described. The particular enzyme of the system combining directly with N_2 has been termed nitrogenase.

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LETHAL ENVIRONMENTAL FACTORS WITHIN THE NATURAL RANGE OF GROWTH

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Perhaps no phase of theoretical biology has received more emphasis than that which deals with the interrelationships of the organism and its environment. Such themes as the "struggle for existence," "the survival of the fittest," "the fitness of the environment," and "the continuous adjustment of internal relations to external relations" are all rooted in this same central problem.

While the vicissitudes of the environment are recognized as of utmost importance in the struggle for existence, the fact that fluctuations well within the natural range of growth of the organism may prove lethal to the young of the species has not been generally recognized. The proverbial delicacy of the "hot house plant" is well known; and the common practice of "hardening" young greenhouse plants, by subjecting them to temperatures a little above the freezing point, has been shown experimentally to protect against freezing conditions which the "unhardened" plant cannot endure. So far as we are aware, however, it has not been shown that environmental fluctuations within the range necessary for growth may be fatal to plants. In the animal kingdom, a clear-cut recognition of the fact exists in the case of fish. The so-called "tempering" process, in which a change from warm to cold water is made gradually, is a common practice in hatcheries, and is supported by an abundance of experience. Scientific proof of this fact is furnished by the work of Loeb and Wasteneys (1912) on *Fundulus*. It was shown that fish transferred suddenly from water at 10° to 35°C. died after a short exposure, whereas if they were changed first from 10° to 27°C., after a few days at the latter

temperature the change to 35°C. could be made without fatal results. In bacteriology the principle has been entirely missed, though it is quite possible that supporting data, obtained in unrelated experiments, lie buried in the literature.

In a study of the physiology of young bacterial cells, Sherman and Albus (1923) observed the lethal effects of mild environmental factors such as low temperatures and dilute salt solutions, which have no measurable destructive action on mature cells. It was then pointed out as probable that the unfavorable fluctuations of a natural habitat lead to a certain mortality among young bacterial cells quite in keeping with that experienced in higher forms of life. Wilson (1922) has concluded from microscopic and cultural studies that, even under constant and apparently optimum conditions, about 10 per cent of the cells of an actively growing bacterial culture are not viable. On the other hand, Kelly and Rahn (1932) were unable to confirm Wilson's results when bacteria and yeasts were observed in microcultures under constant and favorable conditions for growth, the observations continuing through four generations and including 1766 individual cells. The point of issue between these investigators appears to be one of whether or not bacteria, like higher animals, may inherit constitutional weaknesses which lead to death, even under optimum conditions for growth.

It is the purpose of this paper to return to the suggestion of Sherman and Albus that in the struggle for existence the normal hazards of their environment lead to the death of a portion of the young bacterial cells, in the absence of drastic killing factors. It was then shown that young cells perish from exposures which have no apparent effect upon mature cells; evidence that this may also happen within the limits suitable for active growth will be presented in this communication.

II

The strain of *Bacterium coli* used in this study, which is typical of the species, grows actively at 45°C. and also at 10°C. At 45°C. the rate of reproduction is roughly thirty times as fast as at the lower temperature.

When a transfer is made from a young culture in the logarithmic phase of growth at 45°C. to a sterile medium of the same composition at 10°C., there occurs an extensive mortality among the young cells. The data from four such experiments are recorded in table 1.

The growth medium was 1 per cent peptone plus 0.5 per cent sodium chloride with a reaction of pH 7. The numbers of viable cells were determined by the plate culture method, using nutrient agar. All counts reported are averages from triplicate plates. When the transfer was made from 45° to 10°C., 1 cc. was taken from the growing culture and introduced into 100 cc. of the medium at 10°C. The physiologic youth of the cells was doubly ensured by the inoculation of the medium at 45°C. from a previous

TABLE 1

Effect of transfer from 45°C. culture to 10°C. upon young cells of Bacterium coli

EXPERIMENT NUMBER	NUMBER OF BACTERIA PER CUBIC CENTIMETER				PER CENT KILLED (60 MINUTES)
	At beginning	15 minutes	30 minutes	60 minutes	
1	67,000		2,420	2,370	96
2	44,000	1,900	1,540	1,410	97
3	22,000	3,100	1,240	580	97
4	155,000	26,800	18,100	13,500	91

culture in the rapid growth-phase, and by testing the cells before the total count in the culture at 45°C. reached 20,000,000 per cubic centimeter. The experiments were therefore not complicated by an initial "lag" in the test cultures grown at 45°C., nor had they reached the period of "negative acceleration" in growth. The "age" of the 45°C. cultures at time of use ranged from one and one-half to three and one-half hours.

When similar experiments are performed in which young cells growing at 10°C. (cultures one to two days old) are transferred to 45°C., the mortality is less marked. In this case relatively few of the cells are killed during the first fifteen minutes after the transfer is made, and, as is shown in table 2, rapid growth at a rate characteristic of a 45°C. culture is soon initiated.

The fact that the young bacterial cells grown at 10°C. are

more resistant to a change to 45°C. than are those of a 45°C. culture when exposed to 10°C. is probably of considerable biologic interest. A possible explanation for this difference has been suggested elsewhere (Sherman and Cameron, 1933).

TABLE 2

Effect of transfer from 10°C. culture to 45°C. upon young cells of Bacterium coli

EXPERIMENT NUMBER	NUMBER OF BACTERIA PER CUBIC CENTIMETER				PER CENT KILLED (15 MINUTES)
	At beginning	15 minutes	30 minutes	60 minutes	
1	13,700	11,200	18,900	74,300	18
2	4,800	6,700	10,400	31,000	0
3	7,900	5,600	6,100	9,100	29
4	28,000		26,600		

TABLE 3

Effect of transfer from 1 per cent peptone culture to 1 per cent peptone plus 5 per cent NaCl at 40°C. upon young cells of Bacterium coli

EXPERIMENT NUMBER	NUMBER OF BACTERIA PER CUBIC CENTIMETER				PER CENT KILLED (60 MINUTES)
	At beginning	15 minutes	30 minutes	60 minutes	
1	946,000	698,000	487,000	192,000	80
2	156,000	123,000	109,000	44,000	75
3	1,550,000	1,060,000	512,000	361,000	77
4	90,000	56,000	49,100	34,300	62

TABLE 4

Effect of transfer from 1 per cent peptone plus 5 per cent NaCl culture to 1 per cent peptone at 40°C. upon young cells of Bacterium coli

EXPERIMENT NUMBER	NUMBER OF BACTERIA PER CUBIC CENTIMETER				PER CENT KILLED (15 MINUTES)
	At beginning	15 minutes	30 minutes	60 minutes	
1	137,000	12,600		46,000	91
2	28,600	11,000	18,200	33,000	62
3	160,000	77,000	90,000	154,000	52
4	9,600	2,500	5,800	9,100	74

That other environmental changes within the range of growth may be fatal is shown by experiments in which the young cells were grown in 1 per cent peptone and transferred to 1 per cent peptone containing 5 per cent sodium chloride, and vice versa. In

these experiments the cultures were grown at 40°C. and were transferred to the other medium at the same temperature. The results of such tests are given in tables 3 and 4.

When the young cells were changed from 1 per cent peptone to 1 per cent peptone containing 5 per cent sodium chloride, the killing action continued for at least one hour. When the reverse transfer was made, the mortality was greater during the first fifteen minutes, but the surviving cells became adjusted so that reproduction again took place within one hour.

TABLE 5

Effect of transfer from pH 7.0 culture to pH 5.5 at 40°C. upon young cells of Bacterium coli

EXPERIMENT NUMBER	NUMBER OF BACTERIA PER CUBIC CENTIMETER			
	At beginning	15 minutes	30 minutes	60 minutes
1	6,200	8,300	12,300	35,000
2	28,000	23,000	42,000	121,000
3	125,000	176,000	274,000	550,000
4	156,000	176,000	249,000	720,000

TABLE 6

Effect of gradual cooling of 45°C. culture to 10°C. upon young cells of Bacterium coli

EXPERIMENT NUMBER	NUMBER OF BACTERIA PER CUBIC CENTIMETER		
	Initial at 45°C	After reaching 10°C	After 1 hour at 10°C
1	7,200,000	8,400,000	7,900,000
2	716,000	980,000	1,070,000
3	2,000,000	2,430,000	2,320,000
4	287,000	360,000	420,000

It would doubtless be simple to find other types of environmental fluctuations which have lethal effects without going beyond the limits of tolerance for growth. In this connection, however, it is of interest to note that, within the limits tested by us (pH 7.0 to 5.5), abrupt changes in the hydrogen ion concentration of the medium do not have this effect. Table 5 shows no apparent killing action, though a slight lag in growth is indicated.

This result is not surprising. While the temperature of a bacterial cell is that of its environment, it is not to be expected

that the pH of the cell protoplasm is the same as the surrounding medium, nor is it likely that the internal pH would change rapidly. It should be remembered, on the other hand, that *Bacterium coli* grows over a pH range of about 9 to 5, and it is possible that a wider shift in reaction within these limits might have a more drastic effect.

While sudden environmental changes within the limits of growth may be lethal to the young cells, it is of interest to note that if the "shock" is eliminated by gradually bringing about the change, there does not appear to be any killing action. In table 6 are given the data obtained in four experiments in which the medium containing young cells of *Bacterium coli* growing at 45°C. was gradually lowered to 10°C. during a period of thirty minutes.

TABLE 7

Effect of heating at 50°C. for thirty minutes upon young cells of Bacterium coli grown at 10°C.

(See text for explanation)

EXPERIMENT NUMBER	NUMBER OF BACTERIA PER CUBIC CENTIMETER		
	Before heating	Abrupt change: 10° to 50°C.	Abrupt change: 40° to 50°C.
1	289,000	50,000	144,000
2	357,000	78,000	216,000
3	27,600	360	18,500

Bacterial counts were made before the cooling process was begun, when a temperature of 10°C. was attained, and after holding one hour at 10°C.

Another illustration of the sudden shock effect upon young bacterial cells, which is of considerable technical importance to the bacteriologist, has to do with thermal death rates. In such studies it is the usual practice to introduce a small portion of the culture into a larger volume of previously heated sterile diluent, so that a practically instantaneous change is made to the test temperature. When such tests are made with young bacteria, the amount of abrupt temperature change between the incubation and test temperatures becomes important. For example, when young cells of *Bacterium coli* growing at 10°C. are abruptly

changed to 50°C. and held at the latter temperature for thirty minutes, more than 85 per cent are killed. When, on the other hand, the same cells are gradually changed from 10° to 40°C., during a period of ten minutes, and the abrupt change made from 40° to 50°C., only about 50 per cent are killed after thirty minutes at 50°C. These facts are shown in table 7.

III

It is not the intention to enter into a discussion of the theoretical aspects of this work at the present time. Assuming that the same basic facts hold true for higher organisms—and there can be small room for doubt on this point—the general biological implications appear clear.

To the biologist there are here presented fundamental problems dealing with the phenomena of shock and adaptation; and, as is true of many biological problems, the bacteria offer the most elementary, and therefore the best, test material with which to work.

From the standpoint of the bacteria, the struggle with their environment is undoubtedly of great significance. It is clear that mortality among the young is quite as important a problem with the bacteria as among higher forms of life. The rigors of life in a fluctuating environment, aside from selection, may have a "hardening" effect which results in greater viability. Very much to the point, in this connection, are the important findings of Hastings (1923), which showed that bacteria from natural habitats exhibit greater viability than the same organisms when grown in the laboratory on artificial media.

SUMMARY

Young cells of *Bacterium coli* may be killed by abrupt environmental changes within the natural range of growth of the organism.

When young cultures growing at 45°C. are suddenly changed to 10°C., about 95 per cent of the cells are killed within one hour. The bacteria in cultures growing at 10°C. withstand better the sudden change to 45°C. In this case relatively few of the cells are killed, and the survivors soon become adjusted so that rapid growth ensues within one hour.

Drastic killing action is also obtained with abrupt changes in osmotic pressure, without going beyond the limits suitable for growth; for example, transfer from 1 per cent peptone to 1 per cent peptone containing 5 per cent sodium chloride, and vice versa.

While sudden changes within the range of growth have a lethal effect upon the young bacterial cells, there is no apparent killing when the changes are brought about gradually.

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THE USE OF CLARIFIED HONEY IN CULTURE MEDIA

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During the past few years honey agar has assumed an important rôle in the propagation and identification of sugar-tolerant yeasts. Combining the high sugar content and the mineral salts of honey with nutrient agar makes an especially suitable semi-solid medium. In addition to maintaining the desired high osmotic pressure, this combination provides many desirable yeast nutrients.

Fabian and Quinet (1928) reported the use of a honey-agar mixture for the isolation and cultivation of yeasts that cause the spoilage of honey. For their studies honey agar was made by adding one part of honey to two parts of 3 per cent nutrient agar. This mixture, adjusted to pH 6.8 to 7.0, was tubed and sterilized for use as a plating medium and for slants. Marvin (1928), Marvin et al. (1931), Lochhead and Farrell (1931), and others have likewise reported the use of honey agar. Fabian and Hall (1933) successfully extended the use of honey agar to a study of the yeasts encountered in the spoilage of maple sirup.

During the course of an investigation in these laboratories on certain yeasts found in cane sirups and on crystals of white sugar the honey-agar mixture recommended by Fabian and Quinet was employed. Since the colloids of honey bear a positive electric charge and the colloidal agar particles are negatively charged, a flocculation occurs when the two are mixed. The presence of the flocculated materials makes accurate counting of the plates practically impossible (fig. 2) and seriously interferes with the recording of the cultural characteristics of yeast cultures on

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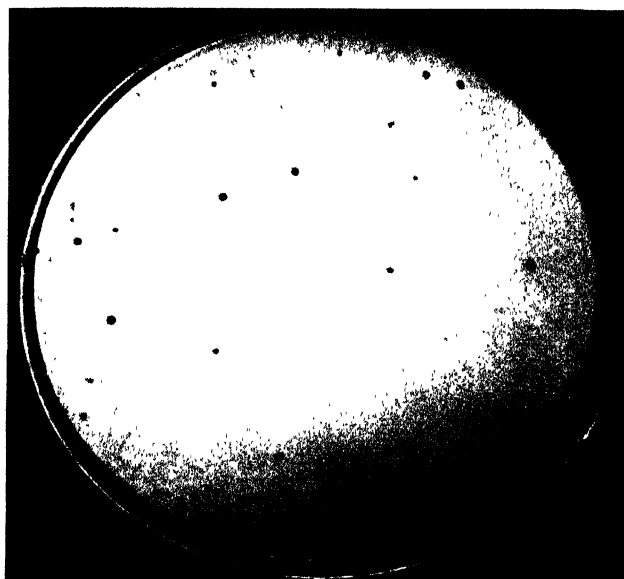


FIG. 1 YEAST CULTURE F-8
Medium: Clarified clover honey agar

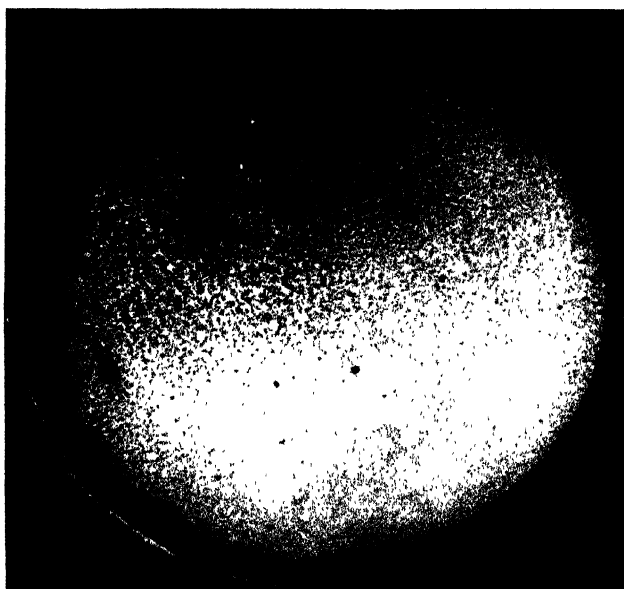


FIG. 2, YEAST CULTURE F-8
Medium: Unclarified clover honey agar

slants. In an attempt to overcome this difficulty honey from which the colloids had been removed by treatment with bentonite was used. The resulting honey agar contained no visible suspended materials and besides permitting the ready examination of the colonies, supported the normal growth of yeast (fig. 1).

REMOVAL OF COLLOIDS FROM HONEY

Colloids are removed from honey by treatment with a suspension of colloidal clay, bentonite.³ For this purpose 750 grams of honey are diluted with about 600 cc. of distilled water, and warmed in a water bath to 50°C., and a 5 per cent suspension of bentonite⁴ is added in the proportion of one volume of bentonite suspension to 15 volumes of honey solution. The whole is thoroughly mixed, and kept at 50°C. for fifteen minutes, after which it is filtered through a large fluted filter. The resulting clear solution is then concentrated to original honey density (80 to 81 per cent solids by refractometer) in vacuo at a low temperature. Two 1-liter distilling flasks connected in the conventional vacuum distillation arrangement are appropriate for this purpose. The clarified honey should be practically free of turbidity.

YEAST GROWTH ON HONEY-AGAR MIXTURES PREPARED FROM CLARIFIED AND UNCLARIFIED HONEY

Since the work of Lochhead and Farrell (1931) indicated that yeast-activating substances in honey are adsorbed by charcoal though not by fuller's earth, the possibility of removing yeast nutrients by treating honey with bentonite was considered. The nutritive values of honey agars made from clarified honey of three floral types (tupelo, fruit bloom and clover) were compared with honey agars made from the same honeys without clarification. Five cultures of sugar-tolerant yeasts were selected for plate and slant studies. For the plate studies a series of dilutions

³ For a more detailed discussion of the behavior of honey colloids, see article by Lothrop and Paine (1931).

⁴ To prepare the suspension, 10 grams of bentonite are added to 200 cc. of water, and the mixture is stirred rapidly with a motor-driven stirrer until a thin creamy paste is formed.

of the yeast cultures were made in physiological saline solution. Tubes of the clarified and unclarified honey agar were seeded (after having been melted and cooled to 45° to 50°C.) with 1 cc. of the dilutions of the yeast suspension. The seeded honey agars were then plated in sterile petri dishes and incubated at 30°C. for several days. At the termination of the incubation period, usually four to five days, total plate counts were made, and the diameters of a representative number of the surface and subsur-

TABLE 1

Comparison of yeast colony counts and average diameters of surface and subsurface colonies developing on clarified and unclarified honey agars

CULTURE	TYPE OF HONEY	DAYS INCUBATED AT 30°C.	PLATE COUNT		AVERAGE COLONY SIZE			
			Clarified	Unclarified	Clarified		Unclarified	
					Surface	Subsurface	Surface	Subsurface
					mm.	mm.	mm.	mm.
F-8	Tupelo	4	128	154	2.45	1.67	2.18	1.85
F-9	Tupelo	4	256	237	3.40	2.60	3.00	3.30
F-10	Tupelo	5	183	183	2.10	1.70	1.98	1.39
F-8	Fruit bloom	4	78	80	1.90	1.81	1.95	1.78
F-9	Fruit bloom	4	154	139	2.07	2.00	2.13	2.04
F-9	Fruit bloom	5	203	207	2.37	1.96	2.16	1.78
F-10	Fruit bloom	4	243	260	1.87	1.69	1.93	1.89
F-8	Clover	5	109	97	2.11	1.87	2.30	2.18
F-9	Clover	5	190	211	2.33	2.24	2.12	2.00
F-9	Clover	5	156	137	2.21	1.84	2.29	1.90
F-10	Clover	5	236	218	2.00	1.51	2.07	1.51
F-11	Clover	4	21	20	1.72	1.63	1.80	1.59
F-12	Clover	4	36	54	1.56	1.34	1.60	1.27

face colonies were determined. The results of these determinations are recorded in table 1.

The results indicate that the total numbers of colonies developing on plates poured with clarified honey agars and on those poured with unclarified honey agars are equal, within the limits of accuracy for dilution plate methods. There were slight variations in the average diameters of the colonies on the clarified and on the unclarified honey agars. These variations appear to be

due to differences in the depth at which the colonies developed, and were observed for all types of honey.

Honey-agar slants were streaked with the yeast cultures for a macroscopical comparison of the amounts of growth and also the

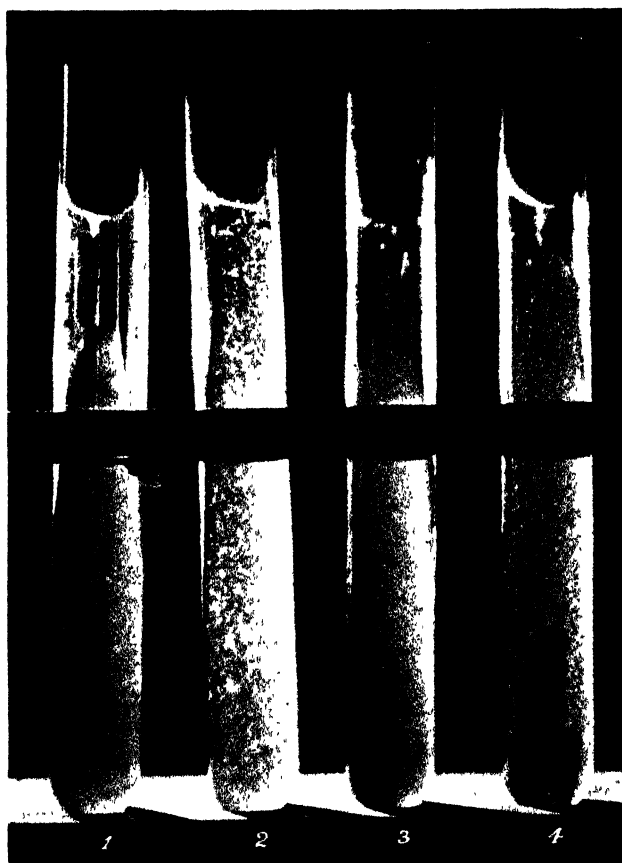


FIG. 3 No. 1 Clarified alfalfa honey agar. No. 2 Unclearified alfalfa honey agar. No. 3 Clarified fruit bloom honey agar. No. 4. Unclearified fruit bloom honey agar.

cultural characteristics. Heavy suspensions of the yeast cultures were made in physiological salt solution. A 3-mm. loop was immersed in the suspension and was then transferred to the top of the honey-agar slant and streaked toward the butt. This afforded

a standard inoculum and a method whereby the amount of growth on each type of honey agar could be compared. The slants were incubated at 30°C. for four days, after which the results were recorded. The amount of yeast growth on honey-agar slants made with clarified honey approximated that on slants made with unclarified honey. The cultural characteristics of the yeasts did not appear to be altered by growing them on slants of clarified honey agar. Cultures maintained for several weeks on slants of clarified honey agar exhibited the same ability to ferment carbohydrates as did those on slants of unclarified honey agar.

The advantages of the clarification of honey with bentonite suggest the use of this method or similar methods in the preparation of malt extract, fruit and vegetable juices, meat extracts and other types of material used in culture media.

SUMMARY AND CONCLUSIONS

1. The aggregates of flocculated materials occurring normally in honey agar are eliminated by pre-treating honey with bentonite. A practical laboratory method for this treatment is given.
2. Studies were made on honey clarified with bentonite to determine its adaptability to the propagation and identification of sugar-tolerant yeasts.
3. Greater accuracy is obtained with less difficulty in total plate counts of yeasts when clarified honey agar is used as the plating medium.
4. The average size of the colonies which developed on clarified honey agar approximated those which developed on unclarified honey agar, within experimental limits.
5. The recording of cultural characteristics is simplified when clarified honey agar is used as the medium.
6. The amount of growth on slants of clarified honey agar was equivalent to that on slants of unclarified honey agar.
7. The cultural and physiological characteristics of yeasts propagated on clarified honey agar remain unaltered.
8. The use of bentonite or like materials for removing colloids from other ingredients used in culture media is suggested.

Grateful acknowledgment is due Dr. L. H. James of the Food Research Division for his interest in the work and also for photographing the yeast cultures.

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A COMPARATIVE BACTERIOLOGICAL STUDY OF A GROUP OF NON-LACTOSE-FERMENTING BACTERIA ISOLATED FROM STOOLS OF HEALTHY FOOD-HANDLERS

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The bacterial strains reported in this paper were isolated during a routine carrier examination of the stools of a group of normal food-handlers. Though culturally a heterogeneous group, these bacteria are interesting chiefly because of their confusing resemblance to the pathogenic intestinal bacteria when first isolated on differential media. A detailed report of their reactions and possible taxonomic position is considered valuable.

Though unclassified gram-negative bacteria, with slight or no ability to ferment lactose, are frequently encountered in stool examinations, they are, in the majority of cases, isolated from hospitalized groups, or from individuals concerned in outbreaks of intestinal disorders of various kinds. For example, Meinicke and Neuhaus (1909) and Burri and Duggeli (1919), found strains in human feces which were like *B. coli*, but did not ferment lactose, though they could be trained to do so. Many "atypical" cultures were isolated by the British during the last war from soldiers who had attacks of intestinal disease. Douglas (1917), Dobell, Gettings, Jepps and Stephens (1918), and Dean, Adamson, Giles and Williamson (1917) all examined dysentery convalescents who were free of symptoms, and found a total of 130 "unnamed" strains, none of which fermented lactose, and the majority of which produced indol. None agglutinated in paratyphoid sera. Fildes (1917) and Glyn and Robinson (1918) report a total of fifty-one "unknown" paratyphoid-like strains iso-

lated from healthy soldiers. None fermented lactose, almost all produced indol, motility was variable, all fermented glucose, and some fermented sucrose. None agglutinated in paratyphoid A or B sera. Fildes reports eighteen additional strains which were able to ferment lactose after three or four weeks cultivation on this sugar. In 1924, Trawinski, studying a group of typhoid and dysentery convalescents and healthy control subjects, isolated ninety-one strains of intestinal organisms which produced indol, but did not ferment lactose. These cultures did not agglutinate in paratyphoid B serum, and para B did not agglutinate in serum made from a few of the strains. He concludes that these organisms are saprophytes which can become pathogenic for man under the most favorable conditions. Dudgeon in 1926 found 2 per cent of slow lactose-fermenting bacilli in stools of a group of subjects which included typhoid patients, people suffering from dietetic errors, and infections of the respiratory tract, and healthy subjects. He says that such strains "have been mistaken for paratyphoid bacilli, though the indol test distinguishes them." But he concludes that such organisms may give rise to intestinal disorders. Fothergill in 1929 cultured the stools of 104 infants during July to October, most of whom had diarrhea. The organisms he isolated from these cases did not ferment lactose, were non-motile, did not liquefy gelatin, and fermented a considerable number of sugars with acid and gas. Some of his strains finally fermented lactose after seven to ten days incubation. The group was heterogeneous both culturally and antigenically, and evidence for pathogenicity was definite in only two cases. In 1932 Kennedy, Cummings and Morrow described twenty-two strains of slow lactose fermenters which they were unable to classify. Since their group of organisms resembles so closely the strains to be described here, they will be discussed later.

ROUTINE PROCEDURE

The stools of 127 food-handlers were examined in groups of about 25 each week over a period of five weeks during October and November, 1932. A portion of the morning stool was collected

by the subject on a swab, and placed in a test tube containing about 2 cc. of distilled water. The tubes were collected about nine o'clock in the morning, and were brought directly to the laboratory, where the specimens were streaked on differential plates. The stools, in so far as the small specimen would permit description, appeared normal in color and consistency. None indicated a diarrheal condition.

Eosin-methylene-blue agar plates were used as the first differential medium. After twenty-four hours incubation, all colorless colonies were fished to Russell double-sugar agar slants. The growth on the Russell slants was used for Gram stains, agglutination reactions, and for inoculation of the following media: Hiss semi-solid agar for motility determinations, 1 per cent peptone water for indol test, gelatin stabs for liquefaction test, and 1 per cent carbohydrate-peptone water for fermentation reactions. All media were sterilized in the autoclave, sugar-containing media at 10 pounds pressure for twenty minutes, and the other media at 15 pounds pressure for fifteen minutes. The Russell slant cultures were restreaked on eosin-methylene-blue plates to check the purity of the culture, and also to afford an adequate description of the colonies. The cultures were preserved on infusion slants, and were transferred regularly every two weeks. All media were controlled with stock strains of *B. coli*, *B. typhosus*, and *B. paratyphosus* B.

GENERAL CULTURAL REACTIONS

Of the 127 stools examined, 102 were entirely negative, yielding only the common lactose-fermenting bacteria. Twenty-five of the stools, or roughly 20 per cent, produced non-lactose-fermenting colonies resembling pathogenic bacteria. These colonies were few in number. In some cases only 1 or 2 appeared, and never more than 10 or 12 were present on any one plate. *B. coli* was always present in great numbers. In a twenty-four-hour growth these colonies were small, gray in color, and were fairly transparent. When fished to Russell double-sugar slants, the reaction was typical of the paratyphoid group: acid and gas in the butt, and a colorless slant. Two specimens,

strains 38 and 100, produced no gas, resembling the typhoid or dysentery groups. All the cultures were small, gram-negative bacteria, so short as to appear coccoid in form. None of the strains liquefied gelatin in fourteen days. A forty-eight-hour growth on semi-solid agar showed 17 of the 25 cultures to be non-motile, and twenty-one of the strains produced indol. Cultures in infusion broth, and on blood plates, showed the strains to be essentially smooth in character.

Fourteen carbohydrates were used in studying the fermentation reactions. The following results were obtained. None of the strains fermented lactose in twenty-four hours, though some of them exhibited a latent fermentation in two to seven days incubation in 1 per cent lactose. A further discussion of the affinity for lactose will be given later. All the strains fermented a considerable number of carbohydrates with acid and gas production (strains 38 and 100 produced no gas). Glucose, mannitol, maltose and arabinose were universally fermented. Dextrine and xylose were fermented by all but a few strains. Inositol and inulin were not fermented at all, and raffinose was fermented by only two strains. A wide variation was exhibited in the reactions to the other sugars. Table 1 gives the reactions of all the 25 strains after forty-eight hours incubation in the various sugar solutions.

An inspection of the reactions listed in table 1 shows at once that these organisms constitute a very heterogeneous collection. They fall nearer to the colon-paratyphoid series than to any other type of intestinal bacteria, but can be identified completely with neither group. There are seven general groups. A small number of sucrose fermenters are further divided on the basis of dulcitol and salicin fermentation, following the basis of classification of the colon group. The majority of the strains do not ferment sucrose, and these are likewise grouped on the basis of dulcitol and salicin reactions. It will be noted that divisions II, IV and VI each contain two strains which are identical, and groups III and V contain three strains each, which are identical.

Division I contains only one strain, which ferments sucrose and salicin, but not dulcitol. This culture corresponds in this

respect to *B. neapolitanum* of the Colon group, and to *B. guimai* of the intermediate groups of Topley and Wilson (1932), but

TABLE 1
Reactions of strains isolated from healthy food handlers

GROUP	CULTURE NUMBER	LACTOSE	SUCROSE	DULCITOL	SALICIN	GLUCOSE	MANNITOL	MALTULOSE	XYLOSE	RAMNOSE	ARABINOSE	INOSITOL	RAFFINOSE	DEXTRINE	INULIN	MOTILITY	INDOL
I	83	-	AG	-	AG	AG	AG	AG	AG	-	AG	-	-	AG	-	-	+
II	90	-	AG	AG	-	AG	AG	AG	AG	AG	AG	-	-	AG	-	-	+
	17	-	AG	AG	-	AG	AG	AG	AG	AG	AG	-	-	AG	-	-	+
	106	-	AG	AG	-	AG	AG	AG	AG	AG	AG	-	-	AG	-	+	+
III	32	-	-	AG	AG	AG	AG	AG	AG	AG	AG	-	-	AG	-	+	+
	34	-	-	AG	AG	AG	AG	AG	AG	AG	AG	-	-	AG	-	+	+
	42	-	-	AG	AG	AG	AG	AG	AG	AG	AG	-	-	AG	-	+	+
	7	-	-	AG	AG	AG	AG	AG	AG	AG	AG	-	-	AG	-	-	-
	54	-	-	AG	AG	AG	AG	AG	AG	AG	AG	-	-	-	-	-	+
	50	-	-	AG	AG	AG	AG	-	AG	AG	AG	-	-	-	-	-	+
IV	13	-	-	-	-	AG	AG	AG	-	-	AG	-	-	AG	-	-	+
	26	-	-	-	-	AG	AG	AG	-	-	AG	-	-	AG	-	-	+
	62	-	-	-	-	AG	AG	AG	AG	-	AG	-	AG	AG	-	-	+
	63	-	-	-	-	AG	AG	AG	AG	AG	AG	-	-	AG	-	+	-
V	82	-	-	-	AG	AG	AG	AG	AG	AG	AG	-	-	AG	-	-	+
	28	-	-	-	AG	AG	AG	AG	AG	AG	AG	-	-	AG	-	-	+
	142	-	-	-	AG	AG	AG	AG	AG	AG	AG	-	-	AG	-	-	+
	74	-	-	-	AG	AG	AG	AG	AG	AG	AG	-	-	AG	-	-	-
	125	-	-	-	AG	AG	AG	AG	AG	-	AG	-	-	AG	-	+	+
	126	-	-	-	AG	AG	AG	AG	AG	-	AG	-	-	AG	-	+	-
VI	46	-	-	AG	-	AG	AG	AG	AG	AG	AG	-	-	AG	-	-	+
	111	-	-	AG	-	AG	AG	AG	AG	AG	AG	-	-	AG	-	-	+
	10	-	-	AG	-	AG	AG	AG	AG	AG	AG	-	AG	AG	-	+	+
VII	38	-	-	A	-	A	A	A	A	A	A	-	-	A	-	-	+
	100	-	-	-	A	A	A	A	-	-	A	-	-	A	-	-	+

here the likeness ceases. Division II, containing three cultures, ferments sucrose and dulcitol, but not salicin. These strains resemble *B. coscoroba* (which differs from *B. coli-communior* only

in being non-motile), but not completely. This grouping corresponds to no member of the Salmonella group. Division III, with six members, ferments dulcitol and salicin, but not sucrose, which fact suggests *B. immobile* (which differs from *B. coli-communis* only in being non-motile). Further comparison shows the identity to fail from this point on, and the resemblance to the Salmonellas is even less. Division IV has four strains which ferment neither sucrose, salicin nor dulcitol, and which resemble *B. acidi-lactici* in this respect, but in very little else. Division V, with six cultures, does not ferment sucrose or dulcitol, and does ferment salicin. *B. guimai* fits this grouping, but only this far. Division VI has three strains which ferment dulcitol, but do not ferment sucrose or salicin. Several members of the Salmonella group, including *B. paratyphosus* A, *B. paratyphosus* B, *B. abortus-equi*, *B. enteriditis*, *B. suispestifer*, and *B. aertrycke* may be classified in this way, but again complete resemblance is lacking. Strains 38 and 100 are grouped together because of their lack of gas production, but they do not belong to the typhoid or dysentery groups.

In the hope that definite reactions which would identify these organisms more clearly would be elicited by the use of other differential media, the following were employed: lead acetate agar stabs, Jordan's tartaric acid stabs, Endo's agar plates, and Krumwiede's brilliant green agar plates. No consistent reactions were obtained.

Agglutination tests with specific sera showed that none of the twenty-five strains agglutinated in paratyphoid A or B sera in dilutions beginning at 1:100 and continuing up to titre, though seven cultures had given a partial agglutination reaction when mixed on a slide with undiluted serum. Strains 38 and 100, which had produced no gas, were tested with typhoid, Flexner, Hiss and Sonne dysentery sera, and were entirely negative. The conclusion is that none of these strains show antigenic identity with the pathogenic intestinal bacteria which they resemble.

In mid-winter, three to four months after the original isolation of the organisms described, twenty-one of the subjects were recultured, but colorless colonies were recovered from only three

of the stools. For purposes of comparison, the original numbers were used, with the addition of an A. A contrast of tables 1 and 2 will show that strains 26A and 32A differ markedly from their corresponding strains 26 and 32. Stool 100A yielded two types of colony, 100A2, which was identical with the original strain 100, and strain 100A which differed only in producing gas from the sugars fermented. These four new strains were non-motile, produced indol, were gram-negative, coccoid bacteria, which did not ferment lactose. Strains 26A and 32A did not agglutinate in paratyphoid A or B, typhoid or Flexner sera. Strain 100A agglutinated in such low dilution in typhoid serum, and 100A2 so slightly in Flexner serum, that they were considered negative.

TABLE 2
Reactions of strains from repeated stools

STRAIN	LACTOSE	SUCROSE	DULCITOL	SALICIN	GLUCOSE	MANNITOL	MALTOSE	XYLOSE	REAMNOSE	ARABINOSE	INOSITOL	RAFFINOSE	DEXTRINE	INULIN	MOTILITY	INDOL
26 A . .	—	—	—	AG	AG	AG	AG	AG	AG	AG	—	—	AG	—	—	+
32 A . .	—	—	—	—	AG	AG	—	—	A	AG	—	—	—	—	—	+
100 A . .	—	—	—	AG	AG	AG	AG	—	—	AG	—	—	AG	—	—	+
100 A2 . .	—	—	—	A	A	A	A	—	—	A	—	—	A	—	—	+

A brief survey of the reactions of these "atypical" organisms so far shows that they are not only a heterogeneous group, but that they fall into no known grouping of intestinal bacteria. On eosin-methylene blue and Russell double-sugar media, they resemble the pathogenic paratyphoids in appearance, but their fermentation reactions, general indol production, and lack of motility place them nearer to the colon group.

LACTOSE FERMENTATION

Several experiments were tried with media containing lactose to see whether or not these strains would finally ferment this sugar.

Soon after the twenty-five strains were isolated, they were

placed on Russell's double-sugar slants, and were transferred daily for eight days on this medium. Nineteen of the strains did not change in their reactions, giving acid and gas in the butt of the slant only. However, six strains produced acid and gas throughout the slant in from two to five transfers, and in each case the culture then fermented 1 per cent lactose with acid and gas in twenty-four hours. These strains were strains 34, 50, 54, 83, 106 and 111, and it will be noted that they differ in their reactions to the other sugars. All the strains were then put on 1 per cent lactose and were incubated for seven days. Eight strains (34, 42, 50, 54, 62, 83, 90 and 111) fermented lactose with acid and gas in from two to four days, and two others (42 and 100) fermented it with acid only. The fifteen strains which had not fermented lactose were then transferred daily in this medium for fourteen days. Four more strains fermented this sugar with acid and gas in from four to eleven days. These were strains 7, 10, 106 and 125. This makes a total of fourteen out of twenty-five strains which had shown a latent power to ferment lactose.

Five months after the original isolation of these strains, they were all plated on eosin-methylene-blue plates to check the purity and the stability of the cultures. A typical colony was fished from each plate to a Russell slant. These cultures gave the same reactions as they had originally, and were continued on infusion slants as stock cultures which were used in further experimentation with lactose fermentation.

Bronfenbrenner and Davis (1918), in the course of a bacteriological examination of foods, reported the use of higher percentages of lactose to convert to lactose-fermenters some organisms (later identified as *B. coli*) which they had isolated from Endo's plates because they did not ferment lactose. Though their cultures did not react in six days growth in 1 per cent lactose-peptone water, they did ferment 2 and 3 per cent lactose in ten transfers. In accordance with the suggestion obtained from this work, lactose solutions were made up in 1, 2, 3, 5 and 10 per cent concentrations, and tubes of each were inoculated with twelve strains chosen from each of the sugar groups. Each series was controlled with two stock strains of paratyphoid B, one rough and

the other smooth. Cultures were incubated for one week, and reactions were recorded each day.

It was immediately apparent that 5 per cent lactose was more effective in producing a change than the other concentrations. Lower percentages generally took a longer time, and 10 per cent lactose produced irregular results, often with no gas production. In no case did the paratyphoid cultures ferment any lactose solution. Seven of the twelve test cultures fermented 5 per cent lactose with acid and gas in from two to five days. Once any given culture fermented the lactose, it also fermented 1 per cent lactose regularly. When inoculated into Russell double sugar, all the fermenting cultures produced a typical coli reaction with acid throughout the tube, and generous gas production. When plated on E.M.B., four of these cultures produced two types of colonies, one, the original pinkish-gray colony, and the other a typical coli colony, deep blue, with a metallic sheen.

Since all these cultures had been freshly isolated from individual colonies on homogeneous plates, the conclusion must be that dissociation of the culture had taken place in lactose solution, toward a coliform bacterium. This conclusion was further corroborated when the four dissociated cultures were plated again on eosin-methylene-blue following a seven days continuous incubation after fermentation had taken place. At this time the cultures were 99 per cent typical coli, with about 1 per cent retaining the original appearance.

All the other strains, seventeen in number, were then incubated continuously in 5 per cent lactose. The total result was that twelve more strains were converted to lactose fermenters in from one to fourteen days, with the average falling at about four days. These strains all produced coli reactions on Russell double sugar, but exhibited a variety of reactions on eosin-methylene-blue. One culture produced a large per cent of typical coli colonies. The other showed some colonies with deep blue centers and some with the original appearance on each plate. Evidence for dissociation was still apparent, though it was evident that some cultures were more stable than others.

There remained ten strains which fermented lactose very

slightly. These strains were not more amenable to daily transfer than they were to continuous incubation. In one to two days, they all showed a faint acid reaction throughout the tubes, with slight gas production. However, this result contrasts with the action of the paratyphoid B controls which produced no gas, and not the faintest acid reaction at any time. These ten slightly fermenting cultures all produced their original reactions on Russell double sugar and on E.M.B. plates.

This experimentation with lactose fermentation had yielded as its most significant result, the fact that these strains may be definitely excluded from the pathogenic groups, on the basis of their tendency to dissociate toward the coli group. It is suggested that when such organisms are isolated during routine stool examinations, they be placed in 5 per cent lactose solution as the simplest means of determining their characters.

At all events, this group of organisms isolated from healthy food-handlers remains very heterogeneous, even in the ability to ferment lactose with rapidity.

DIARRHEAL STRAINS

During the study of the organisms isolated from the stools of food handlers, eleven already isolated cultures were received from various unrelated sources for further study. These organisms had all been isolated from cases of diarrhea and vomiting of unknown origin. Their reactions are given in table 3. Strains 11F, 12F, 13F, 19F, 22F and 24F came from an epidemic of diarrhea in a tuberculosis sanitarium in November, 1932. Cultures 66F and 68F came from a family epidemic in November, 1932. Strain BF was isolated from an employee, ill with diarrhea and vomiting, in a hospital where two patients had a paratyphoid like fever. Strains CB and CU were from the blood and urine, respectively, of a baby who died of a para-typoid-like fever of three weeks duration in March, 1933. The feces were reported to be an almost pure culture of this organism, and no agglutination took place in para B serum.

When replated on eosin-methylene-blue agar in this laboratory, these strains all produced small, smooth, grayish colonies, some

with faint bluish cast. On Russell double-sugar slants, they all gave a typical paratyphoid reaction: acid and gas in the butt, and a negative slant. All were gram-negative, coccoid bacteria, giving a smooth growth on blood plates, and an even turbidity in broth. All but one culture produced indol, and all but four were non-motile. Gelatin was not liquefied in fourteen days. Inspection of table 3 shows that none of the strains fermented lactose, inositol or inulin. All fermented glucose, mannitol, maltose and arabinose. A considerable variety of reactions are

TABLE 3
Reactions of diarrheal strains

STRAIN	LACTOSE	SUCROSE	DULCITOL	SALICIN	GLUCOSE	MANNITOL	MALTOSE	XYLOSE	RHAMNOSE	ARABINOSE	INOSITOL	RAFFINOSE	DEXTRINE	INULIN	MOTILITY	INDOL
11 F.	-	AG	AG	-	AG	AG	AG	AG	AG	AG	-	-	-	-	-	+
19 F.	-	AG	AG	-	AG	AG	AG	AG	AG	AG	-	-	-	-	-	+
22 F.	-	AG	AG	-	AG	AG	AG	AG	AG	AG	-	-	AG	-	-	+
24 F.	-	AG	AG	-	AG	AG	AG	AG	AG	AG	-	-	AG	-	-	+
66 F.	-	AG	AG	-	AG	AG	AG	AG	A	AG	-	AG	AG	-	-	+
68 F.	-	AG	AG	-	AG	AG	AG	AG	A	AG	-	AG	AG	-	-	+
CB.	-	-	AG	AG	AG	AG	AG	-	AG	AG	-	-	AG	-	+	+
CU.	-	-	AG	AG	AG	AG	AG	-	AG	AG	-	-	AG	-	+	+
13 F.	-	-	-	-	AG	AG	AG	AG	-	AG	AG	-	AG	-	-	+
12 F.	-	-	AG	-	AG	AG	AG	AG	AG	AG	-	-	AG	-	+	+
BF.	-	-	-	AG	AG	AG	AG	AG	AG	AG	-	-	AG	-	+	-

exhibited toward the other sugars. It will be noted that the first group in table 3 corresponds to group II of table 1, and that strains 22F and 24F are identical with strains 90 and 17. It will also be noted that strains 11F and 19F are identical as are 22F and 24F, and 66F and 68F. Strains CB and CU fall into group III of table 1, though they are not identical with any strains in this group. Strain 13F falls into group IV of table 1, but is not identical with any strains in that group. Strain 12F is identical in sugar reactions with 46 and 111 of group VI, but differs in motility and indol. Generally, it can be said that these diarrheal strains resemble more closely the strains obtained from a group of

healthy people than they do any of the accepted groups of intestinal bacteria.

When these strains were tested with specific sera, 12F gave a fine partial agglutination on the slide in para A, para B, and typhoid serum, and when set up in diluted serum, agglutinated partially after a night in the ice box, in para A and typhoid serum in dilution 1:100 to 1:800. Strains 22F and 24F gave a partial agglutination in Flexner serum on the slide, but were negative in diluted serum. All the others were negative in all sera.

In this connection it must be mentioned that stools from twelve individuals suffering from diarrhea of varying severity were plated directly in this laboratory, and none of them produced any suspicious colonies on eosin-methylene-blue agar.

When grown in 5 per cent lactose, these diarrheal strains all fermented it with acid and gas production in from two to seven days. Strains 13F and BF did not give a strong reaction, but fermentation was definite. Fermentation of 1 per cent lactose was also present, in from three to nine days, but four strains did not produce gas in this concentration. When inoculated in Russell double sugar, these strains produced a coli reaction after they had fermented lactose and a definite dissociation into coli was apparent on the eosin-methylene-blue plates. Briefly, the diarrheal strains are very similar in their behaviour to the strains isolated from healthy people.

DISCUSSION

Since failure to ferment lactose is the first criterion for differentiating pathogenic intestinal bacteria from the common inhabitants of the bowel, any colonies which do not ferment lactose require further attention. The literature on intestinal bacteria, as well as several personal communications, reveals that the isolation from human feces of bacteria which can be neither identified nor eliminated by the customary procedure, is not a rare occurrence. The chief importance of these strains in routine bacteriological work lies in their confusing resemblance to the pathogenic forms. For example, Kennedy, Cummings and Morrow (1932) describe 22 strains of slow lactose-fermenters

encountered during routine procedure in a diagnostic laboratory. Their cultures were isolated as colorless colonies on Endo's plates, streaked mostly for routine, carrier, and typhoid stool examination. On the solid differential media, i.e. Endo's medium, Russell double sugar, triple sugar and eosin-methylene-blue agar, these strains resembled the paratyphoid group, but when grown in lactose broth, they all fermented that sugar in from two to fourteen days. Daily transfer in lactose broth speeded up the fermentation reaction. Furthermore, all but one of their strains produced indol. They conclude that these strains must be placed in the genus *Escherichia* since they ferment lactose, but that they can hardly be classified as colon bacilli though "they must be closely allied to that group probably as variants forming one of the connecting links between the colon-aerogenes group, and the paratyphoid group."

The strains described by these authors resemble so closely the strains reported in this paper, that it seems certain that they belong to the same group of bacteria. Variability within the groups in indol production, motility, and carbohydrate fermentation runs parallel. Eight of their strains were classified as *B. coli-mutabile* because of the production of true-breeding red daughter colonies on Endo's plates. When the remaining fourteen strains were compared in cultural reactions with the strains described here, it was apparent that the similarity was very close, though complete identity was lacking. Eleven of their strains fell into several of the sugar groups reported here.

Though the classification of this group of organisms is obviously uncertain, such bacteria must now be recognized as a group characterized by irregular lactose fermentation, and closely related to the colon bacillus.

The presence of these organisms in the feces of a group of normal people is more confusing than their presence in a diseased condition, where they might logically be assumed to be aberrant forms of previous etiological agents in an infection. The problem of what conditions in the intestine favor the loss of the power to ferment lactose, and whether a postulated loss of this power accompanies an increase in pathogenicity, must remain hypo-

thetical for the present. The fact that these organisms were recovered on later examination in only three of the twenty-five original subjects, suggests that they were transient, of no pathogenic significance, and perhaps the result of purely physiological conditions. However, the occurrence of similar strains in cases of diarrhea, indicates that the question is far from settled. It is suggested that the apparent etiological rôle of similar strains in intestinal disturbances of infants may be due to a higher susceptibility in the infant than in the adult. If this be so, we must accordingly admit that such organisms may have a pathogenic effect even in the adult, given the proper intestinal conditions of susceptibility.

It seems highly improbable, in view of the close similarity of these bacteria to other groups reported in the literature, that such organisms constitute a separate, and hitherto unrecognized group of intestinal bacteria. It is more reasonable to assume that we are concerned with a dissociative process in which well defined classes may have produced such variants. In 1928, Dulaney reported the dissociation of *B. coli-communis*, after long incubation in broth, into a rough variant which was colorless on Endo's medium and Russell double sugar, and a smooth variant which produced the customary red reactions on both media. Otherwise the two forms were identical in sugar fermentation. However, a contradictory report was published by Nungester in 1931. He describes the dissociation of a *B. coli*-like organism isolated from a gall bladder empyema, which, after 6 days of continuous incubation in broth, produced two forms, a rough variant which fermented lactose, and a smooth variant which did not attack this sugar. Both forms produced indol. It was possible to change the non-lactose-fermenting smooth variant into a lactose-fermenting organism, but the reverse change with the rough form was not effected. Though it is obvious that the question of dissociation of these forms is still in the realm of conjecture, it is suggested that further work with this group of organisms from this point of view will serve to clarify the subject.

SUMMARY

1. Twenty-nine strains of gram-negative, non-lactose-fermenting intestinal bacteria were isolated from a group of healthy food-handlers during a routine carrier examination.

2. These strains produced non-lactose-fermenting colonies on eosin-methylene-blue plates, and on Russell double sugar, resembling the paratyphoid group.

3. None of the strains fermented 1 per cent lactose solution in forty-eight hours, though they all fermented a considerable number of other carbohydrates.

4. The organisms could be divided into seven groups by sugar fermentation reactions. In some groups there were identical strains.

5. Twenty-five strains produced indol, and twenty-one strains were non-motile.

6. Agglutination reactions with sera of established pathogenic types were negative.

7. Continued growth in 5 per cent lactose solution resulted in varying degrees of dissociation into lactose-fermenting variants, some of which produced typical colon colonies when plated on eosin-methylene-blue agar.

8. The chief importance of these bacteria lies in their confusing resemblance to the pathogenic intestinal organisms on the first differential media.

9. The conclusion is drawn that these strains are closely related to the colon group, possibly as variants, since they tend to dissociate into lactose-fermenting coli-like organisms.

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THE VARIANT AND FILTERABLE FORMS OF CERTAIN GREEN-PRODUCING STREPTOCOCCI¹

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Interest in the problem of bacterial variation during the last decade has brought the heterogeneous group of green-producing streptococci under scrutiny. Studies of the reciprocal relationship between colony form (rough or smooth) and virulence, antigenic structure, opsonic index, sensitizing property, varying cataphoretic potentials and the production of filterable forms have brought forward much new knowledge of variation within this group of organisms. In many of these reports the occurrence of morphologically variant cells is mentioned as an incidental observation. Such variant cells are often diphtheroids, frequently diplococci and occasionally swollen, nodular rods. The origin of the distinct cell types, their essential properties, their interrelationships, and their fate have not been clearly recorded.

Pleomorphic green-producing streptococci upon primary isolation and after extended cultivation exhibit chains of flattened diplococci of greatly varying sizes, huge coccid and constricted forms and spindle-shaped rods. The unstable form of the organisms suggests that such pleomorphic strains may be especially suitable for the study of the origin of variant cells, their distinctive properties and relationships, as well as of the occurrence of filterable forms in streptococci.

Sixty-one strains of highly pleomorphic green-producing streptococci were isolated from human throats during the early,

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active and convalescent stages of acute upper respiratory infections occurring in Chicago from December, 1928, to February, 1931. One exceptional strain, No. 42X, was isolated from the blood culture of a patient during the early stages of influenzal infection. The characteristics of this strain (with its smooth and rough substrains) have been described at length in a previous report (Falk, Harrison, McKinney and Stuppy, 1931); therefore it has been used as a standard of comparison for the remaining strains.

NATURAL HISTORY OF THE STREPTOCOCCAL STRAINS

Swabs passed over the reddened fauces, tonsils and pharynx were streaked on plates of heated blood agar and hemolyzed blood agar.² The latter transparent medium was preferred since it clearly revealed the colonial structure by *transmitted light* which simplified greatly the technic of isolation. The predominating organisms on the plates were green-producing streptococci of many colonial types. On every plate on which pleomorphic streptococci were found, another organism growing in gray mucoid colonies or in wrinkled colonies was conspicuously present. This was a Gram-negative diplococcus which in cultural reactions did not conform wholly to the Neisserian types listed by Bergey. This is no new observation (Jordan, 1927; Cooper, 1929; Traut and Herrold, 1930; Falk et al., 1931; Coburn, 1931); whether it represents a chance association, a "satellite phenomenon," or a commensal relationship has not been determined.

The colonies of pleomorphic streptococci on all solid mediums were extremely minute. Unfortunately, they possessed no characteristic colonial form by which the strains could be certainly recognized in a mixed streptococcal flora. All gradations of colonies ranging from definitely smooth colonies to markedly rough colonies were found on the original plates. The smooth colonies were very minute, round, raised and glassy yellow; the intermediate colonies were somewhat larger than the smooth colonies, round, raised and glassy yellow surrounded by a whitish

² Hemolyzed blood agar: 2 per cent sheep blood laked in water added to veal infusion agar, pH 7.8, at 60° to 70°C., giving a clear, reddish-brown agar.

halo of streptococci growing in a single plane (pl. A, fig. 1). The halo was the first visible evidence of dissociation from the smooth to the intermediate state. The roughest colonies were flat, gray, coarsely granular, with or without a central depression and irregular edge (pl. A, fig. 2).

Occasionally on the primary plates rough, grooved colonies were found which had little effect upon the blood but which left their exact impress in the agar—a mirror image of the grooved or capitate form of the exposed surface of the colony. The organisms in such colonies were swollen rods and huge cocci. At times colonies which powerfully yellow-bleached the medium were found to contain Gram-positive diplococci. The solid blood medium immediately surrounding the colonies at first was rendered green by the growing colonies (6 to 8 hours); after twenty-four hours this area had a light yellow or “bleached” appearance. Diphtheroids were found in small gray or yellow mucoid colonies—these latter often mottled with flaky material, edge irregular, lacy or raised (pl. A, fig. 3). The word pleomorphic is a term describing a wide variety of cellular shapes. There was no constant correlation, however, between the colony type—rough or smooth—and distinctive cellular morphology, the character of the medium and age of the culture. Under no experimental conditions were the individual cells of either rough or smooth colonies of streptococci entirely non-pleomorphic.

Biochemical characteristics

None of the strains hemolyzed 5 per cent sheep or human blood agar, but several strains hemolyzed 5 per cent rabbit blood agar when tested by the Brown method. All strains were insoluble in a 10 per cent aqueous solution of “Difco” bile.

Fermentation of carbohydrates was tested in 1 per cent filtered Pfanstiehl sucrose, lactose, salicin, raffinose, inulin and mannitol added to sugar-free veal infusion broth, pH 7.8, indicator brom-cresol-purple. Some of the more complex carbohydrates were utilized very slowly. All of the strains fermented sucrose; none of them utilized mannitol. One non-lactose fermenting rough strain was found. Of the 62 strains, 33 (53 per cent)

fermented salicin; 44 (70 per cent) fermented raffinose; 19 (30 per cent) fermented inulin and of these 19 strains, 4 also fermented salicin and raffinose.

Proteolytic action was studied in Loeffler serum by the test of Wollman, Urbain and Ostrowsky (1922). A day-old culture of streptococci in a liquid medium containing serum was successively inoculated with *E. coli*. The latter organism produced indol if the albumin of the medium had been utilized by the streptococci. There was no evidence of proteolytic action with either medium.

The test of Greenthal (1930) was used to detect the reduction of nitrates to nitrites. A few crystals of nitrite-free sodium nitrate were dropped in an eighteen to twenty-four hour broth culture of streptococci. A few drops of 10 per cent sulphuric acid, potassium iodide and dilute starch were added in succession. None of the pleomorphic streptococci thus tested gave a positive reaction.

As the fresh strains were isolated from the throat cultures they were used as antigens in agglutination tests with stock rabbit antiserum against the strain 42X (rough). Sixteen strains agglutinated by a serum dilution of 1:128 or more were selected for later work.

The mediums and the conditions evoking variation

First, many experiments were performed to determine the effect of a wide variety of mediums and conditions of growth in effecting variation in rough and smooth strains of pleomorphic streptococci. Second, when morphological variants of one form or another were found in a culture, single cell isolations were made to secure strains for more detailed experiments. Space forbids a complete account of all experiments whether effective or not; therefore only a concise summary of apparently successful procedures and results will be given.

The mediums used were:

1. Heated or hemolyzed sheep blood agar containing 2 per cent Witte peptone.
2. Veal infusion broth (double strength), pH 7.8, without salt, or peptone. (This is referred to subsequently as veal infusion broth no. 1).

3. Veal infusion broth no. 1 with 0.5 per cent mannitol.
4. Veal infusion broth no. 1 with 10 or 30 per cent anti-streptococcal rabbit serum with a demonstrable titer against homologous or heterologous strains.
5. Five per cent soluble potato starch in Tyrode's solution.
6. Cultural association with *L. acidophilus* in veal infusion broth no. 1 with 0.5 per cent glucose.

Method

The method found most generally satisfactory was as follows: selected strains (3 smooth strains and 3 rough strains) were transferred from fresh preliminary cultures on hemolyzed blood agar to a series of the test mediums. These were incubated at 37°C. for twenty-four hours, then at room temperature thereafter for twenty days. Smears and transfers to hemolyzed blood agar plates were made daily from each tube. Uninoculated tubes of medium were treated similarly as controls.

Results

1. Transfers of smooth or intermediate colonies of streptococci from blood or glucose broth to 2 per cent Witte peptone hemolyzed blood agar resulted in *rough*, proteolytic, feebly green-producing colonies containing bulging and constricted pleomorphic rods. These rods were impossible to stabilize on any medium and, particularly, in broth, and reverted quickly to the streptococcal form. Serial transfer on 2 per cent Witte peptone blood agar produced a smooth-to-rough colony transformation. Chamot and Georgia (1925), Davis (1927), and McAlpine and Brigham (1925) agree that Witte peptone is markedly different in growth-promoting properties and in chemical composition from other widely used peptones. The latter workers report that Witte peptone is rich in protein nitrogen with a low content of non-protein nitrogen and amino nitrogen in contrast to similar analyses of other brands. This agar then, with a high content of Witte peptone and blood, may be considered an "over-rich" growth-promoting medium.

2. In veal infusion broth, pH 7.8, without salt, peptone, glucose or blood—a poorly buffered "starvation medium" with a relatively high surface tension—the streptococcal chains increased

rapidly in length and in pleomorphism of individual cells. After forty-eight hours there were branching chains (pl. A, fig. 4), lateral and transverse fission of the cocci with lateral budding and a "fading out" of the formed coccal elements in the chains (pl. A, fig. 5). After six to sixteen days the chains were long, frequently branched sheaths containing randomly disposed blue-staining material no longer in the coccal form (pl. A, figs. 5, 5a, 5b). Viable transfers from strains in this medium reproduced green-producing colonies containing either streptococci or diplococci. There was no change in the inoculated rough or smooth colony form.

In the broth just described combined with 0.5 per cent mannitol the streptococcal chains soon fragmented to 4 to 8 coccal portions. In older cultures (seventy-two to ninety-six hours) three-cell division, lateral budding and discrete diplococci were commonly found. In certain cultures large faintly staining globoid bodies appeared; these cultures after seven to ten days reproduced on transfer to solid mediums gray, non-green-forming colonies containing short polar-staining diphtheroid organisms. There was a gradual change from smooth and rough colonies to the intermediate colony type.

4. In veal infusion broth, pH 7.8, containing no other ingredients except one-third volume anti-streptococcal rabbit serum, certain strains of streptococci were reduced to cocci and diplococci in an amorphous mass containing tiny stainable granules (pl. A, fig. 6). In these cultures no chain formation was seen after seventy-two to ninety-six hours; nor were the large inflated bodies found. Transfers from such cultures after varying periods of incubation very occasionally produced mucoid yellow colonies containing metachromatic staining diphtheroid rods (pl. B, fig. 6) or frequently gray intensely green-producing colonies of Gram-positive flattened diplococci. In the heterologous strains affected by this medium the titer ranged from 124 to 512; the titer against the homologous strain was 1024. The rough strains of streptococci in this medium were unchanged in colonial appearance; the smooth colonies on later transfers were topped with raised yellow caps—a precursor of a smooth-to-intermediate colony transformation.

5. In the soluble starch-Tyrode medium the definitely rough strains were rapidly and progressively converted to smoother strains. The chains grew to a moderate length with little pleomorphism. Then the chains fragmented with later Y-formed chains. After ninety-six to one hundred and twenty hours incubation, diplococci were present in the smears, and transfers to hemolyzed blood agar resulted in many reactive greening or "bleaching" colonies of Gram-positive diplococci, scattered among colonies containing green-producing streptococci.

6. The *L. acidophilus* and streptococcal strains were grown separately in the veal infusion broth for twenty-four hours, then combined for subsequent incubation in a flask. Lateral budding and short chain formation were found in nearly all combined cultures after twelve to twenty-four hours incubation. Massed diplococci alone remained of the streptococcal chains after three to six days. Streptococcal colonies were found on transfer to the eighth day; colonies containing diplococci only appeared on the fourth to sixth daily transfer and continued in some strains to the twelfth daily transfer. The *L. acidophilus* colonies were ignored. This lytic action on the streptococcal strains was not due solely to the lactic acid or metabolic products of the associated growth, for filtrates of seventy-two-hour cultures of *L. acidophilus* alone added to several twenty-four-hour cultures of streptococci had no similar effect. This observation confirms a similar one made by Schiller (1914).

No organisms were found in the uninoculated tubes either in the smear or by transfer.

Summary

The primary object in these and many other experiments was to effect a definite change in morphology in a known streptococcal culture. When streptococcal strains were passed by rapid serial transfer on a single favorable medium, changes in colony form did occur, but the pleomorphism was moderate and no variants formed. When, however, streptococcal organisms were *aged* in an unfavorable or a very stimulating medium there often occurred atypical cell division, branching chains, "fading" of formed cocci, swollen deeply staining bodies at intervals in the

chain and terminally, lateral budding, and finally complete reduction of the chains to an amorphous mass containing diplococci and granules. Transfers from such *aging* cultures to mediums of a different physical and chemical state resulted in colonies differing from the streptococcal colony and containing distinct variant cells: a diphtheroid, a diplococcus or a spindle-shaped rod.

FILTERABLE FORM FROM STREPTOCOCCI

It is an interesting fact that the mediums and methods employed for preliminary cultivation by most of the workers reporting filterable forms of streptococci are precisely those known to stimulate variation.³

The evidence up to this point has indicated that the production of pleomorphic elements, atypical cell division, lateral budding, branching and the reduction of chains to an amorphous mass preceded the formation of variants from a streptococcal culture. The three mediums in which a rapid chain reduction was most conspicuous were selected for preliminary cultivation preceding filtration experiments.

Method

Both rough and smooth strains of streptococci on heated blood agar were composed of moderately long chains of organisms occurring typically in pairs of flattened diplococci. These varied irregularly from minute organisms to elongated, inflated elements often centrally constricted. All gradations of size

³ Ramsine (1926)—streptococcal toxin prepared in glucose sheep-blood broth and cultural association with a related strain of streptococcus in 10 per cent sucrose broth.

Sedaillon and Gaumond (1927)—guinea pig peritoneal exudate, also whole blood.

Hauduroy and Leabre (1927)—peptone water.

Urbain (1927)—5 per cent soluble starch.

Rashkowaka (1928)—Martin's broth with 20 to 25 per cent ascitic fluid.

Riccitelli (14)—ascitic fluid broth.

Hadley, Delves, and Klimek (1931)—passage in lithium chloride broth; also in pancreatin broth.

Evans (1932)—rabbit brain passage.

Richardson (1932)—rabbit brain or peritoneal passage.

and shape of cocci occurred in the unbroken chain. Here and there in the chain areas appeared not retaining the blue stain and which had the appearance of a sheath no longer containing rounded stainable cocci.

Six strains (3 rough and 3 smooth) of pleomorphic streptococci were each inoculated into 8 tubes of the following mediums:

1. Ten per cent 42XS immune rabbit serum with veal infusion broth No. 1.

2. Five per cent soluble starch in Tyrode's solution.

3. Cultural association with *L. acidophilus* in glucose veal-infusion broth.

At the end of 6, 12, 24, 48, 72, 96, 120 and 144 hours incubation, a tube of each series was examined by smear, then filtered with a saline suspension of a fresh culture of *B. prodigiosus* as control with an equal amount of uninoculated medium. On alternate days as a further control, incubated but uninoculated mediums were also filtered and incubated with the test filtrates. The cultures containing *L. acidophilus* were controlled in addition by a separate filtration of *L. acidophilus* alone and incubated with the test cultures.

The filters were Seitz-Werke Germicide filters, Manteufel model, assembled without the narrow metal ring supplied by the manufacturers. The filtrate was received in a 10-by-1-inch tube placed in a liter filter flask. All filtrations were made with reduced water pressure, in most cases checked by a gauge. Serum-containing mediums were filtered at 100 to 120 mm. Hg pressure; the starch-containing medium was filtered at 150 mm. pressure. Filtrations often were completed in 4 minutes and always within twenty minutes. The large tube containing the filtrate was removed with aseptic precautions, plugged securely with a plug from a similar tube, covered with tinfoil and incubated at 37°C. for at least three weeks unless growth was indicated before that time.

Summarizing, filtration was performed when certain morphologic changes had occurred in the culture filtered. The entire filtrate was incubated in one tube and not dispensed to several kinds of medium. Additional similar medium equal in volume to

the filtrate was filtered with the culture to add to the nutritive properties of the liquid.

Results

The first evidence of growth in a filtrate under these conditions was not an opalescence, for the fluid remained clear, but a fine, barely perceptible precipitate which settled on the glass to the surface of the liquid. At first there was no precipitate in the bottom of the tube. As incubation continued the density of the precipitate increased yet the fluid remained clear. The first indication of a precipitate appeared between the eighth and twelfth days of incubation. When the tube on tilting presented a distinctly "ground glass" appearance it was opened for the *first time*.

The precipitate was always adherent to the glass and could not be shaken loose; it was most easily removed by scraping with a sterile Pasteur pipette with which a generous amount was transferred. Secondary growths from the clear liquid were never obtained; if at all, they were secured from transfers of the adherent precipitate.

About 0.1 cc. of the loosened precipitate was discharged from the pipette to the surface of a plate of 2 per cent Witte peptone heated-blood agar, and spread over a small area in the center of the plate. Twenty-four hours later 1 cc. of glucose broth was transferred to the same area, the moistened surface scraped with a loop, and the liquid re-transferred to another similar plate *seriatim* until a green reaction on the medium or colony formation indicated growth.

Usually after 5 or 6 serial passages there was a faint green reaction and when growth was definitely established the organisms "yellow-bleached" the medium. In smears from primary subcultures the organisms were diplococci often of uneven size. In stabilized cultures the organisms were diplococci, strongly Gram-positive, with flattened apposed sides.

The salient data derived from the filtration experiments were:

1. Diplococci were obtained from filtrates of cultures in

a. Immune serum broth: (1) from the homologous smooth strain; (2) from an heterologous rough strain.⁴

b. Starch-Tyrode medium: (1) from one smooth strain; (2) from two rough strains.

c. L. acidophilus association: from two smooth strains.

2. Viable elements appeared in the filtrates of those cultures *only* which were reduced to an amorphous state or a diplococcal form *before* filtration. This required a preliminary incubation of ninety-six to one hundred and forty-four hours.

3. The filtrates of cultures retaining the streptococcal form to the moment of filtration were sterile.

4. The filtrate upgrowth was slow (eight to twelve days) and had a marked attraction for glass—a characteristic in no way resembling cultural growth preceding filtration.

5. The organism recovered from the filtrate did not resemble the streptococcus cultured before filtration in morphology or biologic characteristics.

6. Control organisms *filtered with* the test culture did not pass the filter.

SINGLE CELL ISOLATION

In the technical manipulations recorded, the question may be raised whether the organisms described—the spindle shaped rod, the diphtheroid and diplococcus—were real variants of streptococci or were mere contaminants. While the cultural experiments were in progress it became equally necessary to develop a technic for the isolation of single cells for more detailed study. After preliminary trials, the technics advocated by Gee and Hunt (1929), and by Avery and Leland (1927) were combined. The chamber and technic described by the first authors were used to isolate the single cells; the chamber of the second authors was used as an incubating chamber.

This chamber was a brass ring 35 by 3 by 1 mm. cut from a brass tube, with short arms attached. On one side the chamber was completed by a 45- by 50-mm. No. 1 cover slip ruled into

⁴ From a third rough strain a diphtheroid was obtained from the filtrate after 12 serial passages.

minute squares (after paraffining) with a fine needle and hydrofluoric acid. The ring was dipped into hot paraffin and placed on a sterile etched slip in a petri dish. Witte heated blood agar, clarified while hot, was added to the well by pipette.

Single organisms were transferred by micro pipette (cf. Gee and Hunt, 1929) to the agar and the chamber completed and sealed firmly by dipping the free rim in hot paraffin and inverting on a 3- by 1-inch slide. The chamber was examined immediately with the oil immersion objective, the presence of single cells verified and their location plotted by means of the ruled squares. After incubation transfer was made from discrete colonies.

The swollen rods from proteolytic colonies and the diphtheroids from yellow mucoid colonies were not easily miscible in water, saline or broth. Shaking such cells in Tyrode's solution freed enough for isolation purposes. The diplococci were readily dispersed in broth. The spindle-like rods and the diplococci had a vitality as single cells far exceeding that of the diphtheroids. Of attempts to isolate cells from 18 diphtheroid strains only 7 single-cell diphtheroid strains were obtained on the Witte blood agar.

The spindle shaped rod

The unstable bulging and tapered rod was repeatedly found in primary transfers from the throat to heated or hemolyzed blood agar. In established streptococcal cultures the rod appeared in transfers from aging cultures on Loeffler serum or from blood broth to 2 per cent Witte peptone heated-blood agar. When the bulge or node was most evident there was also to be found (preferably by carefully made and stained contact preparations) a lateral "budding" from the nodular portion of the rod. Two or even three "buds" may be seen, each attached to the rod by a fine stalk (pl. B, fig. 1).

A rough strain of streptococci after six days incubation in glucose blood broth at room temperature was transferred to clarified 2 per cent Witte peptone heated-blood agar. Rough colonies developed interspersed with extremely minute colonies

the latter colonies detectible only with a long focus binocular colony microscope (pl. B, fig. 2). A contact preparation made by pressing a sterile cover slip on the growth was fixed in formol-Zenker solution, then dehydrated and stained with the Churchman modification of the Gram stain. The organisms in some of the larger rough colonies were spindle shaped rods with lateral budding (pl. B, fig. 3). In other colonies the rods were nodular and elongated containing granules arranged in pairs (pl. B, fig. 4). The organisms in the minute colonies were indefinitely stained granules and rods (pl. B, fig. 5). The organisms in the rough colonies quickly reverted to streptococci in broth cultures; the organisms in the minute colonies after 8 subcultures in semi-solid buffered brain agar, one transfer on Loeffler serum and finally in glucose broth became streptococcal in form.

The large, rod-like form (in massive and in single cell cultures) quickly merged into streptococci in liquid or on solid substrates. The development of the rod to the streptococcus was as follows:

A. The spindle-shaped rod, sharply tapered at either end, often constricted in its widest portion. At the pointed ends minute cocci appeared, which, dividing by simple fission, started a chain formation.

B. The nodal rods showed a regularly dispersed metachromatic stained condensation of chromatin alternating with deeply stained nodes. The stained granules lying in pairs side by side in the sheath appeared to round up as cocci. These dividing by simple fission formed a chain of pleomorphic streptococci.

The rod stage could not be stabilized, but while in this stage the organisms were definitely proteolytic on solid mediums. They had little or no effect upon blood pigments. There was no demonstrable change in sugar utilization or in nitrate reduction from that found in the corresponding streptococcus.

Cornil and Babes (1890), Arloing and Chantre (1894), Krasowska and Nitsch (1918), Brosq et al. (1923), Urbain (1927) and Euler (1927) observed such rod-like forms in primary cultures from body membranes or fluids in mediums containing blood or serum. All of these workers commented upon the fact that in such cultures the organisms within twenty-four to forty-eight

hours elongated with nodal swellings and evolved into typical streptococci. Other observers of this form in streptococcal cultures have recorded the branching of the streptococcal chains either preceding or following the development of the rod stage (Seitz (1896), Stolz (1898), Lorenz (1909), and Fry (1919)).

It is possible that the pleomorphic rod under discussion is similar to the "spore producing" rod of Evans (1926; 1927) isolated from encephalitic virus material or virus-inoculated brain emulsions in cooked meat medium, and more recently described as evolving from streptococci (Evans, 1932). It must be added, however, that the motility of the rods and the unmistakable spore formation described by Evans were not observed.

The diphtheroid

The sequence of colonial and morphologic change observed in the origin of a diphtheroid from a single-celled (growth from three connected coccal elements) rough strain of streptococci, No. 42XR, follows:

A transfer of the strain to veal infusion broth containing one-third volume of immune serum against a related strain, No. 389, was incubated at 37°C. for twenty-four hours and at room temperature for ten days. A transfer to hemolyzed blood agar produced discrete rough colonies interspersed with minute yellow mucoid colonies. The organisms in the mucoid colonies were large irregular coccal bodies, some of which seemed to contain two rods in a faintly staining "shell" (pl. B, fig. 7).

Two uninoculated tubes of the serum broth treated as the test culture gave no evidence of growth.

The yellow mucoid colony on serial transfer produced no green reaction on hemolyzed blood agar (pl. B, fig. 6). The smear was composed of metachromatic-staining curved and clubbed rods (pl. B, fig. 8). A return from this rod to the streptococcal stage was obtained by alternate transfer on fresh Loeffler serum with condensation water and glucose veal-infusion broth, forty-eight to seventy-two hours elapsing between transfers. This method of the alternate use of solid and liquid mediums, with a prolonged period between transfers, was the most successful method used in

inducing the diphtheroid strains to show streptococcal relationships.

Diphtheroids in gray colonies repeatedly occurred after prolonged incubation of streptococci, usually at room or icebox temperatures, followed by transfer to a different medium. In five instances such gray diphtheroid-containing colonies resulted in transfers to a dissimilar medium from aged single-celled strains of diplococci (of streptococcal origin). The yellow mucoid type of colony appeared in transfers from prolonged incubation in a serum-containing liquid medium or in primary transfers from the throat.

The diphtheroid appeared to arise from streptococcal or diplococcal antecedent cells by an enlargement of certain cocci in which chromatin possibly was reorganized, reproducing upon transfer as rods. As these rods merged into the streptococcal form the metachromatic-staining material first condensed at certain points in the rod, then rounded up as cocci which reproduced as streptococci by simple fission.

The biochemical characteristics of such diphtheroid strains in general were: a limited and slow utilization of sucrose, lactose, maltose and dextrin; no effect upon blood pigment; moderate proteolytic action (yellow strains); and slight reduction of nitrates to nitrites (especially chromogenic strains).

Investigators who have reported diphtheroids in known streptococcal cultures have used for the most part body fluids or mediums containing such fluids (Lemoine (1896), Mellon (1917), Urbain (1927), and Ramsine (1927). The diphtheroids occurred only after considerable aging of the streptococci in such mediums.

Jensen and Morton (1931) isolated a streptococcal strain which on blood agar was consistently diphtheroidal in form and in glucose brain-broth as constantly grew as streptococci. The two phases were found to differ in virulence, cataphoretic potentials, peroxide production and serologic specificity. Koch and Mellon (1930) support the view that diphtheroids from blood cultures may at times be the attenuated, non-virulent phase of virulent streptococci. Thompson (1932), however, in a large

series of blood cultures found no evidence of a genetic relationship between diphtheroid and streptococcus.

The diplococcus

As a variant form in streptococcal cultures the diplococcus appeared to take form in two ways: directly by lateral budding from cocci in the intact streptococcal chain; and indirectly, following more or less complete disintegration and dissolution of the chains.

The formation of diplococci indirectly in liquid mediums was preceded by a branching of the chains, then a slow, cumulative

TABLE 1

MEDIUMS	INCUBATION PERIOD	MORPHOLOGY
1. Heated blood agar.....	6 days	Diplococci
2. Veal inf. broth, pH 8.2, 0.5 per cent glucose	96 hours	Diplococci
3. 2 per cent Witte peptone blood agar, no salt	48 hours	Diplococci
4. Ascitic fluid broth, pH 8.0	72 hours	Diplococci
5. Ascitic fluid agar, pH 7.8	48 hours	Huge and minute cocci
6. Loeffler's serum.....	48 hours	Clubbed rods
7. Hemolyzed blood agar, 2 transfers	48 hours	Slight green reaction
8. Veal inf. broth, pH 7.8, 0.5 per cent glucose	24 hours	Streptococci

fading of the formed coccal elements. The randomly-dispersed blue-staining material for a time retained the stain within a faintly pink-staining streptococcal sheath and then the chain formation completely disappeared (pl. A, fig. 5a). Following this, smears revealed only Gram-positive cocci and diplococci in a pink amorphous mass containing minute faintly-staining granules (pl. A, fig. 6). *This granule formation is stressed.* Transfers from this terminal stage to solid mediums frequently produced diplococci only and not streptococci.

Filtration, if performed after the terminal granular stage had been reached, was useful in separating viable elements which on subculture resulted usually in diplococci and, less often, in diphtheroids. Such diplococci obtained from cultures and filtrates

were single-celled and resultant growths were carried in stock on heated blood agar.

Two single-celled filtrate strains of diplococci, 42XS-d, derived from a filtrate of No. 42XS in homologous immune serum broth, and 389-d, derived in a similar manner from homologous immune serum broth, were cultured to determine whether such diplococci were truly related to the original streptococci. The 42XS-d diplococcus returned to the streptococcal form after transfers through the sequence of mediums shown in table 1.

The recovered streptococcus fermented sucrose, lactose and salicin completely (pH 5.4) and raffinose slightly (pH 6.0); the original streptococcus failed to ferment raffinose but fermented the other sugars. The original streptococcus was agglutinated by its homologous antiserum in a dilution of 1:1024, the recovered streptococcus was agglutinated by this serum in a dilution of 1:128; there was no agglutination of the intermediate diplococcus.

The diplococcus 389-d after aging twenty-two days in mannitol broth was incubated seventy-two hours on a hemolyzed blood agar (made with a filtrate of a broth culture of the streptococcus 389). The organisms in the flat, concentric, feebly green-producing colonies were huge and minute diplococci. Some of the larger cocci appeared to be budding (pl. A, fig. 7). Two transfers on 2 per cent Witte heated-blood agar followed by veal infusion glucose broth produced the streptococcal form.

The original streptococcus, No. 389, failed to ferment salicin and mannitol, the recovered streptococcus failed to ferment mannitol. The antiserum homologous for the original strain No. 389 agglutinated the organisms in a dilution of 1:512; the recovered streptococcus was agglutinated in a dilution of 1:64; the diplococcus was not agglutinated.

The diplococcus (of streptococcal origin) possessed two striking biologic characteristics:

1. The diplococcus had a far more pronounced action upon blood than the streptococcus; it "yellow-bleached" blood agar whereas the streptococcus on the same plate produced a moderate narrow green zone.

2. The diplococcus in liquid mediums strongly reduced nitrates to nitrites. This property was not possessed by the corresponding

streptococcus. The diplococcus was not bile-soluble and feebly fermented five of the six sugars tested.

The essential points in the literature of pleomorphic streptococci over three decades were tabulated in abstract by Donaldson (1922). Many strains when first seen on artificial mediums were diplococci, and the term "diplo-streptococcus" was a common characterization of pleomorphic streptococci.

Löhnis (1921) emphasized the stability of variant cocci and diplococci from streptococci and other bacterial species. Once stabilized by various technics the organisms grew readily and were resistant to change. The same point was re-emphasized by Hauduroy (1929) and by Hadley and collaborators (1931). Nevertheless, the transformation of diplococci *from* streptococci back *to* the streptococcal stage has been recorded by Lorenz (1909), Taddei (1909), Mellon (1911), Euler (1927), and Lautier (1929).

ANTIGENIC RELATIONSHIP OF STREPTOCOCCAL AND VARIANT STRAINS

The low-titered agglutination of the diphtheroid variant by rabbit serum prepared against the undissociated streptococcus and the total absence of agglutination of the variant diplococcus by this serum indicated the need for a more complete record of the serologic interrelationship of the three forms: streptococcus, diphtheroid and diplococcus.

Rabbits in duplicate were immunized with each of the following antigens representing two distinct strains:

- | | | |
|---|---|--|
| A | { | Rabbits (2) immunized with the original rough streptococcal strain No. 389. |
| | | Rabbits (2) immunized with No. 389 diphtheroid from an eight-day culture in mannitol broth. |
| | | Rabbits (2) immunized with No. 389 diplococcus from a filtrate of streptococcus No. 389 grown in homologous immune serum. |
| B | { | Rabbits (2) immunized with original smooth streptococcal strain No. 42XS. |
| | | Rabbits (2) immunized with 42XS diphtheroid (yellow mucoid) from a transfer from a forty-six-day blood broth culture to heated blood agar. |
| | | Rabbits (2) immunized with 42XS diplococcus from a filtrate of No. 42XS in homologous immune serum broth. |

389 series

Agglutination tests were set up between each antigen of the No. 389 series and each immune serum, controlled by similar tests with normal serum of the immunized rabbits and the usual saline controls. The antigens in the control tests were not agglutinated.

42XS series

A similar test was made with the antisera of the 42XS series of antigens. Two sets of the serial dilutions of sera were prepared; to one set were added living antigens washed from

TABLE 2

Agglutinations of strain No. 389, and its variants by their respective antisera

ANTISERUM AGAINST	ANTIGEN TESTED	AGGLUTINATION FINAL SERUM DILUTION
389 streptococcus	389 streptococcus	1,024
	389 diphtheroid	64
	389 diplococcus	None
389 diphtheroid	389 diphtheroid	512
	389 streptococcus	32
	389 diplococcus	64
389 diplococcus	389 diplococcus	1,024
	389 diphtheroid	32
	389 streptococcus	None

twenty-four-hour agar cultures with 0.8 per cent NaCl solution. To the other set were added stock antigens used for immunization and diluted with saline to the required turbidity.

The antigens in the control tests were not agglutinated. The diphtheroid antigen appeared to be partially lysed in the first 3 or 4 tubes of the dilution series by both the streptococcal and diplococcal antisera. The agglutinative titers with the diluted stored antigens of the same series were similar except that the seeming lytic effect against the diphtheroid antigen by the streptococcal and diplococcal specific sera was not evident.

In these and similar tests the diphtheroid antigen was agglutinated not only by its homologous antiserum, but also to some degree by the serums against the streptococcus and the diplococcus. Both the streptococcus and the diplococcus were agglutinated perceptibly by the diphtheroid antiserum.

It thus appears from the evidence of the serologic tests that the diphtheroid may be a form intermediate in antigenic structure between the streptococcus and the diplococcus. The sequence in morphologic changes in cultures also indicated this relationship. When streptococci were derived from diplococci they passed

TABLE 3

Agglutinations of strain No. 42XS and its variants by their respective antisera

ANTISERUM AGAINST	ANTIGEN TESTED	AGGLUTINATION FINAL SERUM DILUTION
42XS streptococcus	42XS streptococcus	1,024
	42XS diphtheroid	128
	42XS diplococci	None
42XS diphtheroid	42XS diphtheroid	512
	42XS streptococcus	64
	42XS diplococcus	32
42XS diplococcus	42XS diplococcus	1,024
	42XS diphtheroid	64
	42XS streptococcus	None

almost without exception through an intermediate diphtheroid stage before again merging into the streptococcal form. The one exception to this generalization was a diplococcus from a filtrate growth which on subculture merged directly into a streptococcus by a preliminary lateral budding preceding active simple fission (pl. A, figs. 7, 8, 9).

DISCUSSION

The pleomorphic green-producing streptococcus removed from its natural habitat—the living body—is composed of flattened diplococci in chains interspersed with enlarged, constricted bodies.

Huge cocci, diplococci and rods, both spindle-shaped and diphtheroid, complete the varied microscopic picture.

The streptococcus as well as three of the morphologic elements—the diplococcus, the diphtheroid and the spindle-shaped rod—occurred discretely in separate culture. The spindle-shaped rod was formed especially on solid mediums favoring growth; its existence as such was brief for it quickly elongated by fission to a chain. A prolonged incubation of streptococci in mediums limiting growth and multiplication, followed by transfer to mediums of a different physical state and chemical composition, produced variant colonies containing variant cells—the diphtheroid or the diplococcus. Once formed these cells were maintained by rapid transfer on the same mediums on which they arose.

Visible changes occurred in the streptococcal cultures preceding the terminal growth of variants. Atypical cell division produced branched chains or discrete cocci; the chain sheath disintegrated and finally cocci and granules only remained of the original culture. Granule formation was always present in liquid cultures in which, after filtration, a secondary growth developed. The diplococci recovered from such filtrate growths may have originated from (1) diplococci passing by chance a faulty filter or (2) few or many potentially coccil forming granules.

One-half of the volume of the incubated filtrate contained the metabolic products of growing and dying organisms; the remainder—fresh medium filtered with the culture—presumably furnished sufficient nutriment to permit the development of a filter passing form. It cannot be denied that since diplococci were present in the medium before filtration certain organisms may have passed the filter and slowly reproduced themselves. Against this argument may be placed these observations: (1) in filtering simultaneously diplococcal and granule-containing cultures with *B. prodigiosus* in no instance was there a re-growth in the filtrate of the control organism; (2) the filtrate growth developed very slowly and had a marked attraction for glass—a characteristic quite unlike that of broth cultures of single cell diplococci which produced a turbid growth in forty-eight hours; (3) repeated sub-

culture on solid mediums was required before the filtrate growth assumed the diplococcal form and attributes.

The granule may be merely a lifeless product of bacterial metabolism or it may be a specialized physiologic unit carrying in the most condensed form the latent characteristics of the strain. Löhnis (1921) compiled many observations of granule formation by bacteria and discussed the phenomenon in relation to regeneration of the usual bacterial cell. Hadley et al. (1931) in agreement with the interpretation of Löhnis, recently, discussed further the possible relation of granule formation to filter passing forms of the Shiga bacillus and related species. Kendall (1931; 1932) cultured several species of bacteria in a special protein medium. He examined his cultures with the ultramicroscope immediately before and after filtration and found granules which he interpreted as living filterable forms. However formed, the diplococcus from streptococcus filtrate growths possessed biochemical functions quite distinct from those of the streptococcus cultured for filtration. Repeated changes in environment induced in two strains of filtrate diplococci morphologic variations resulting finally in streptococci similar in attributes to the original streptococcal strains.

The chief interest of the cultural results lies in the evidence, under the conditions studied, of the range of variable form and function by which streptococci perpetuate themselves. Further study is suggested in regard to chemical analyses of the antigenic constitution of the original and variant organisms, and observation of their effect upon living cells by animal inoculation or by combining them with normal and specifically immunized tissue cultures.

CONCLUSIONS

1. Rapid serial transfers of rough or smooth green-producing streptococci upon a single medium induce a change in colony type but *no change* in morphology. Streptococci aged in unfavorable or in stimulating mediums followed by repeated transfers to mediums of differing physical and chemical states produce variant colonies containing morphologically variant cells.

2. Pleomorphic green-producing streptococci give rise by described technics to three variants—a blood-bleaching, nitrite-forming diplococcus, in rounded, moderately rough colonies, a diphtheroid in gray or yellow mucoid colonies, and a proteolytic spindle-shaped rod in definitely rough colonies.

3. The diplococcus and the diphtheroid possess an antigenic structure and biochemical functions differing from each other and from those possessed by the original streptococcus. Diplococci and diphtheroids so formed give rise again to streptococci by a process which is preceded and probably depends upon a changed mode of cell division. The streptococcus thus recovered resembles, but is not identical with, the original streptococcus in serological properties and biochemical action.

4. A precipitate containing viable forms occasionally develops in the filtrates of streptococcal cultures. This occurs when atypical cell division, chain reduction and granule formation is clearly evident in the culture before filtration.

5. The viable forms eventually by a suitable technic assume a visible form, in most cases a diplococcus, similar to the diplococcus isolated from the same culture before filtration. In two cases diplococci from the filtrates were converted to streptococci with attributes similar to but not identical with those of the original streptococcal strains.

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PLATES

PLATE A

FIG 1 Intermediate colony. $\times 100$

FIG 2 Markedly rough colony. $\times 150$

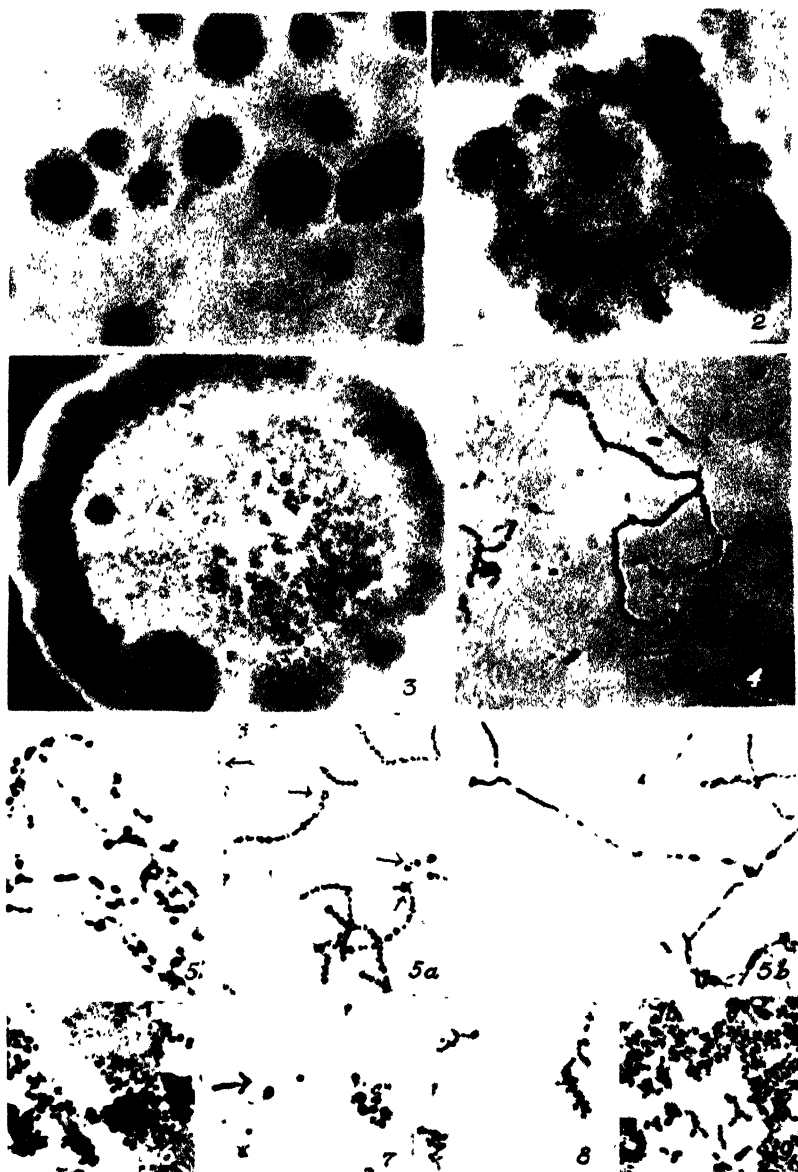
FIG 3 Mucoid colony flecked with yellow material containing diphtheroids.
 $\times 200$ All colonies on hemolyzed blood agar, forty-eight hours

FIG 4. Rough strain in veal infusion broth, twenty-four hours. Note branching.

FIGS. 5, 5A AND 5B. Same culture after six to ten days. Streptococcal sheath still intact. $\times 1000$

FIG 6 Smooth strain in 10 per cent homologous immune serum broth six days. Diplococci and granular material. $\times 1000$

FIGS, 7, 8 AND 9. Filtrate diplococcus. Lateral budding of cocci and transitional forms to streptococci. $\times 1000$.



(Ruth A. McKinney: Forms of Green-producing Streptococci)

PLATE B

FIG. 1. Contact preparation of culture on 2 per cent Witte heated blood agar, twenty-four hours. Lateral budding from swollen rods. $\times 2000$.

FIG. 2. Rough strain on clarified Witte blood agar; large and minute colonies. $\times 150$.

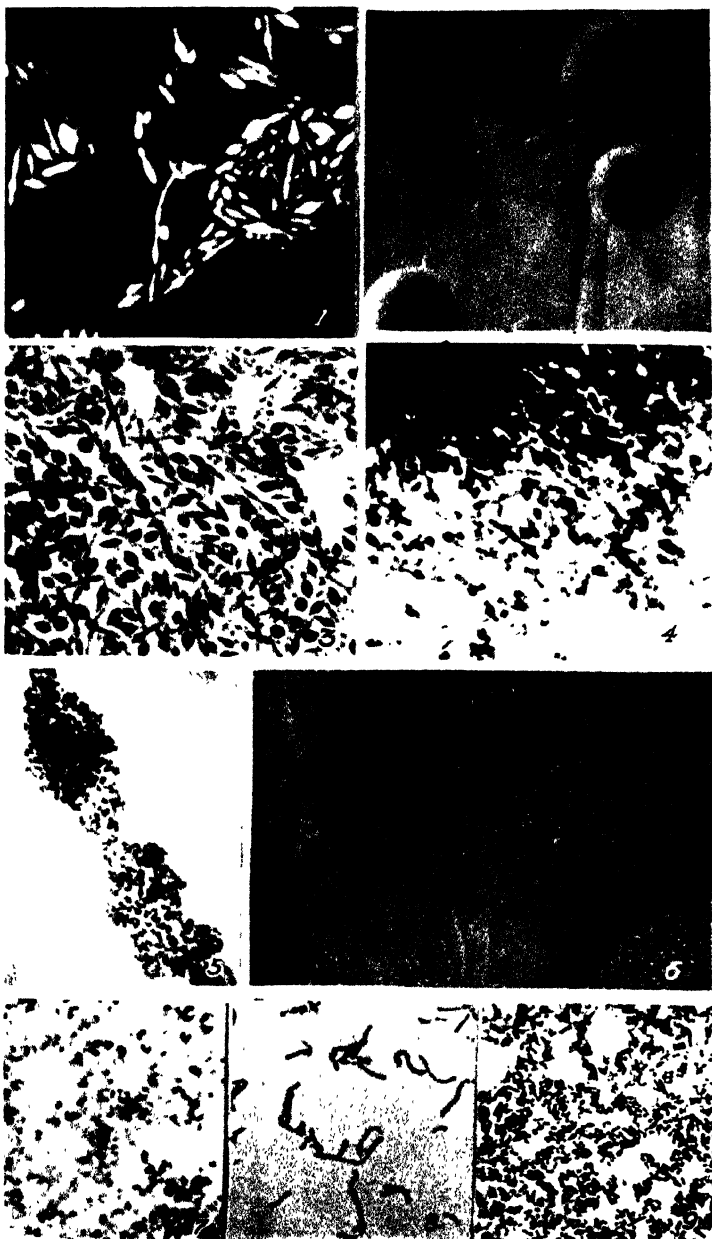
FIGS. 3 AND 4. Stained contact preparation of large colonies in figure 2. Spindle-shaped rods with lateral budding; nodular metachromatic staining rods. $\times 1200$.

FIG. 5. Stained preparation of minute colony, fig. 2. $\times 1200$.

FIG. 6. Yellow mucoid variant colonies from rough streptococcus. $\times 950$.

FIGS. 7 AND 8. Organisms in mucoid yellow colony, a variant of a rough streptococcus. $\times 1000$.

FIG. 9. Smear from other variant gray diphtheroid containing colony. $\times 1000$.



(Ruth A. McKinney: Forms of Green-producing Streptococci)

I. A COCCOID FORM OF *C. DIPHTHERIAE* SUSCEPTIBLE TO BACTERIOPHAGE¹

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INTRODUCTION

In the last fifteen years, bacteriophages active against a number of organisms, including *C. diphtheriae*, have been observed. D'Herelle, in 1918, obtained two races of bacteriophage from the feces of two horses immunized by injection of cultures of *C. diphtheriae*. This bacteriophage was active only against atoxic strains of the bacillus. In 1921, Botez claimed to have obtained lysis in series using *C. diphtheriae*, pseudodiphtheria organisms, *B. dysenteriae*, and *B. anthracis*, by introducing into broth cultures of the organism one loopful of a saturated alcoholic solution of methyl violet. D'Herelle (1926) was later unable to confirm these results.

In 1924, Blair obtained a lytic principle active against avirulent *C. diphtheriae*, (a) from the intestinal contents of guinea pigs inoculated with *C. diphtheriae*, (b) from the peritoneal exudates of such guinea pigs, and (c) from a diphtheria culture two months old. Fejgin (1925) also obtained a lytic agent from an avirulent culture of *C. diphtheriae* in Martin's bouillon that had stood for several weeks.

Klosterman and Small (1928), working only with toxic strains of *C. diphtheriae*, attempted to isolate an antidiphtheria bacteriophage, (a) from stools of typical cases of diphtheria, (b) from a thirty-three-day-old broth culture, and (c) from the intestinal and peritoneal washings of guinea pigs inoculated with the organ-

¹ This work has been supported by a grant from The Chemical Foundation, Inc., of New York City.

ism, but obtained negative results. From eleven specimens of feces, obtained from eleven antitoxin horses, they obtained one race of bacteriophage active against three strains of *C. diphtheriae*, but not active against the Park-Williams No. 8 strain. Six of the horses from which no bacteriophage was obtained had been immunized with this No. 8 strain.

In 1931, Smith and Jordan reported having obtained a race of bacteriophage active with respect to *C. diphtheriae*. Using a culture of a virulent organism isolated from a typical case of diphtheria, and a sewage filtrate as source of bacteriophage, growth of a certain strain (Wright Strain) was inhibited. Repeated contacts of the filtrate of known potency for the Wright strain and other strains were made in an attempt to adapt the lytic agent to different strains, the results being negative. From various sources including stools from diphtheria patients, they obtained numerous races of bacteriophage, for which only one susceptible strain of the organism was isolated.

Since bacteriophage against *C. diphtheriae* has been obtained from so many sources, isolation of other susceptible strains and a study of the form in which they are susceptible seemed important.

EXPERIMENTAL

Nose and throat cultures and stools from a three-year-old child having a typical case of laryngeal and tonsillar diphtheria were obtained.² The disease started with a cold and sore throat, a hoarse croupy cough later developing. On admission to the hospital ten days after onset of the disease, there was diffuse redness of the throat with a grayish membrane. The tonsils were large, edematous, and red, with flecks of grayish yellow membrane. The larynx was red and moderately edematous with a tag of membrane extending into the trachea. The pharynx was apparently clean. Cultures from the throat and larynx were positive. The patient had received 5000 units of antitoxin before admission to the hospital. After admission, he was given another dose of 10,000 units of antitoxin intramuscularly and 10,000

² Obtained through the courtesy of Dr. Lawrence Smith of the Willard Parker Hospital, New York City.

units intravenously. Recovery followed with no complications and the patient was discharged eleven days later. The culture and specimen of stools used in our study were obtained four days before he was discharged from the hospital.

The initial throat culture (RB-2T) streaked on Loeffler's serum agar showed typical Gram-positive rods after twenty-four hours incubation (37°C.). A virulence test in guinea pigs was positive. Blood agar plates were streaked, and a pure culture of these rods was isolated. From then on, daily transplants into veal-peptone broth were made. The broth was prepared as follows:

1. 1 pound veal (Bacto) 7 liters water
2. Heated 50°C. for thirty minutes
3. Brought to boil, boiled for one to two minutes
4. Filtered through a towel
5. Added 0.5 per cent c.p. NaCl
 2 per cent proteose peptone
6. Adjusted to pH 7.6 with NaOH
7. Boiled five minutes
8. Filtered until crystal clear
9. Tuber
10. Sterilized, 15 pounds pressure for fifteen minutes

After several transplants in broth, which had been tested for sterility, a pure culture of coccoid forms was obtained from the pure culture of *C. diphtheriae* (RB-2T) which had previously shown the characteristic rod forms. These coccoid forms were transplanted onto Loeffler's slants and stored in the icebox for five weeks. On subsequent transfer into veal-peptone broth, short typical rods appeared and the culture remained in this form for a considerable period of time. Eventually the coccoid forms reappeared. As the culture became better adapted to the media, it stayed in the typical rod stage for shorter periods of time. The tendency of a culture to revert to either the rod or coccoid form was usually observed in a single culture, in which the two forms co-existed in varying proportions. We have observed this change in other cultures of *C. diphtheriae*, which were obtained from typical cases of diphtheria and in the P. W. No. 8 strain, when grown under similar conditions. We, therefore, feel justified in assuming that the morphological changes in *C. diphtheriae*

(RB-2T) are not peculiar to this strain but may be characteristic of any typical diphtheria culture. By fishing colonies from blood agar plates, we attempted to isolate a culture of typical diphtheria rods that would not revert to the coccus form, but were unsuccessful. We therefore considered this to be a pure culture (fig. 3, 1.)

A sterile filtrate of the stools of the same patient was obtained by filtration through paper and through a Berkefeld N filter.

Using this culture of *C. diphtheriae* (RB-2T), and a filtrate of the stools obtained from the same patient, repeated contacts of the filtrate with the organisms were made in an attempt to demonstrate the presence of bacteriophage. After eight transfers, lysis was observed in broth, and after ten transfers, it was observed on 1 per cent agar plates containing ascitic fluid.

Other races of bacteriophage active against this strain of *C. diphtheriae* were obtained from the peritoneal fluid and intestinal contents of guinea pigs that had been injected intraperitoneally with 1 cc. of a twenty-four-hour broth culture. The presence of these races of bacteriophage was not observed until after two to three transfers of the sterile filtrates in contact with the organisms.

Cultures of this strain were grown for four weeks at room temperature, in veal-peptone broth. They were then filtered through Berkefeld N filters and weak races of bacteriophage, active against the specific organisms, were obtained. Filtrates of cultures, prepared with 0.15 per cent glucose and 0.3 per cent maltose, according to the method of Hazen and Heller (1931), after two to six weeks incubation at 37°C., were likewise active against this particular organism.

Nose and throat cultures and stools from four other cases of diphtheria were obtained. Pure cultures were isolated as before and again both rods and cocci appeared after several transplants on veal-peptone broth. The same was true of the P. W. No. 8 strain.

By the three methods described above, we obtained races of bacteriophage active against two of these strains of *C. diphtheriae*. In no case was cross lysis observed. In the same way, filtrates

of low lytic potency for the P. W. No. 8 strain were obtained. The No. 8 strain used was highly toxic when the work was started. After transfers in veal-peptone broth for several months, its ability to produce toxin had been partially diminished. It was for this less toxic strain that we were able to produce active bacteriophage.

As criteria for bacteriophagy (Topley and Wilson, 1931), we used certain facts established by the work of Twort, and of d'Herelle. (1) The bacteriophage must be separable from the bacterial cells by filtration through a Berkefeld N filter. (2) It must cause partial or complete lysis at some stage of bacterial growth. (3) It must be possible to propagate the bacteriophage in series in the presence of living, actively reproducing, susceptible bacterial cells.

To demonstrate lysis, the following technic was used.

1. Liquid media: Broth tubes were inoculated with one loopful of a twenty-four-hour culture of *C. diphtheriae* plus one loopful of the lytic filtrate. If bacteriophage was present, the tubes were clear at the end of eighteen hours incubation at 37°C. At the bottom of the tube, there remained a floccular sediment which on staining showed granular material produced by the lysis of the organisms. At times the broth appeared opalescent or cloudy, and on staining showed coccoidal forms. Controls inoculated with no bacteriophage grew with pellicles and on staining showed typical organisms. Whether these coccoidal forms represent secondary growth or whether they represent resistant forms as d'Herelle (1926) has stated, we cannot say. However, they represent a form of cocci different from those that are lysed completely by the bacteriophage (fig. 3, 2, 4).

2. Solid media: a. Plaque method: Approximately 0.05 cc. of bacteriophage plus 0.05 cc. of a twenty-four-hour culture of the bacilli were spread with a glass rod on a 1 per cent agar plate containing ascitic fluid. Care was taken to mix the bacteriophage and the organisms thoroughly, and to spread the mixture in such a way that the entire plate was covered with the inoculum. After eighteen hours incubation at 37°C. areas were observed on the plate where no growth had occurred. This indicated that a bacteriophage was present which had lysed the susceptible organisms.

b. Streak method: An ascitic agar plate was streaked with a young

culture of the organism, and after one hour incubation (37°C.) one drop of the lytic filtrate was placed on the streak. If bacteriophage was present, heavy growth was observed along the line of inoculation, the point that had been in contact with the bacteriophage showing considerably less or no growth after eighteen hours incubation at 37°C.

We were later able to demonstrate lysis more clearly by using veal-peptone broth containing 1 per cent agar.

Various methods were used in an attempt to increase the titer of the bacteriophage:

A. According to the procedure of Keller (1931), an equal volume of a one-hour culture was added to a certain number of cubic centimeters of bacteriophage. The one-hour culture was prepared by inoculating one loopful of a five-hour culture into 6 cc. of broth and incubating for one hour at 37°C. If the broth was clear after twenty-four hours at room temperature, an equal volume of the one-hour inoculum was again added. If after twenty-four hours, the broth appeared opalescent, it was allowed to stand for seven days; it was then filtered, and an equal volume of the one-hour inoculum added. If the mixture was turbid after twenty-four hours, it was filtered after four days at room temperature. A quantity of the inoculum equal to one-half the volume of the original mixture was then added to this filtrate. By repeating this procedure for several weeks, we were able to increase the volume of the bacteriophage without decreasing its potency, but were unable to increase the potency appreciably.

B. Partial desiccation *in vacuo* was attempted as a means of concentrating the bacteriophage. There was no increase in the potency as far as could be observed.

C. Larger amounts of more potent bacteriophage were best obtained by inoculating flasks containing 100 cc. of veal-peptone broth with 1 cc. of a weak race of bacteriophage plus 4 cc. of the specific organism and incubating at 37°C. for two to three weeks. The potency of the bacteriophage was increased, and there was an indication that the presence of bacteriophage causes an increase in the amount of toxin and porphyrin (Coulter and Stone, 1931) produced by the organisms.

D. Our attempts to concentrate the bacteriophage led us to the

question of its purification. Clifton, in 1930, reported having purified bacteriophage by adsorption with $\text{Al}(\text{OH})_3$ and separation from the alumina mass by $(\text{NH}_4)_2\text{HPO}_4$. In accordance with his method, 10 cc. of a suspension of bacteriophage was stirred for one hour with an equal volume of a 3.5 per cent suspension of aluminum hydroxide. The alumina mass was collected on a Buchner funnel, washed five times with distilled water, and then stirred for a half-hour with 10 cc. of a 0.5 per cent suspension of secondary ammonium phosphate. This mixture was incubated overnight at 37°C ., filtered with suction, and the bacteriophage recovered in the filtrate. Flocculation of this filtrate, according to the Ramon method showed that the Lf of the toxin had been diminished considerably, and therefore that most of the toxin had been destroyed or separated from the bacteriophage. The titer of the bacteriophage had not been increased.

At this point, we noticed that the degree of susceptibility, of the RB-2T strain, to lysis varied with the morphological form of the organisms. The organisms passed from the typical rod stage through various forms to a coccoid stage, returning to the rod form every three to five days. The degree of susceptibility of the culture to the bacteriophage seemed to vary directly with the number of cocci present.

By means of the Chambers micromanipulator (1922-3), single cells were isolated from this strain (RB-2T) in an attempt to obtain a completely susceptible culture. By fishing a single coccus, inoculating it into broth, and incubating twenty-four to forty-eight hours at 37°C . we were able to obtain cultures completely susceptible to the homologous bacteriophage (figs. 1, 7 and 3, 2). By serial transfers of the susceptible culture with the lytic filtrate, the titer of the bacteriophage was increased to 10^{-6} .

So far we have been unable to keep these single-cell coccoid cultures in a completely susceptible form for more than six to eight days at a time. Any slight morphological change seems to decrease the susceptibility to lysis. Extremely small cocci, giant cocci, coccoidal rods, short solidly-staining rods and granules usually appear between the coccoid stage and that in which typical granular or barred rods are present.

On isolation of single rods, cultures were obtained that were completely resistant to the bacteriophage. In no case were we able to keep this culture in the rod form for more than two to three transfers. The rods changed, usually within twenty-four hours, forming coccoidal bodies, which soon became true cocci, susceptible to bacteriophage. After a few transplants, they lost their susceptibility, and eventually returned to the rod stage (fig. 3, 3), only to change again to the coccoid form.

Although this strain shows a definite change from rods to cocci, and back to rods, we have no evidence that there is a definite life cycle. The intermediate steps in the transformation of the rods into cocci, or vice versa, vary. The coccoid form is extremely hemolytic for rabbit's red blood corpuscles (fig. 1, 5). It produces a large raised white colony, which is smooth, glistening, and of mucoid consistency (fig. 1, 6). Veal-peptone broth is clouded uniformly by this form, a slimy mucoid pellicle being produced. The rod form is non-hemolytic, and produces a smaller grayish-white colony (fig. 1, 4; note that this colony appears larger than that in figure 1, 6, due to greater magnification), which is intermediate between the mucoid colony and the typical No. 8 colony (fig. 1, 1).

DISCUSSION

It is generally recognized that bacteriophages for various organisms are present in the intestinal contents of infected persons or animals, in the peritoneal washings of such animals, and in aged broth cultures. We have confirmed the work of Smith and Jordan (1931), in which they showed that antidiphtheria bacteriophage can be recovered from these same sources, and from aged toxins as well. Only one of their strains was completely susceptible, however, and even this strain decreased in susceptibility after serial transfers in broth.

Bacteriophage multiplies only in the presence of living, actively-reproducing, susceptible bacterial cells. Therefore it would seem likely that from each source of bacteriophage it should be possible to isolate an organism which is susceptible to this specific bacterio-

phage. In this study, we have worked with four throat cultures from diphtheria cases, and with the P. W. No. 8 strain. Races of bacteriophage were obtained against 4 of the 5 strains. Partial lysis of three of the strains was observed, whereas one strain showed complete lysis. The fact that we have isolated a completely susceptible strain in only one case, and then only by single-cell isolation of a certain morphological form, indicates that the organism is susceptible to bacteriophage only during one stage in its growth. It is not unlikely therefore that every strain of *C. diphtheriae*, at some stage in its development, passes through a susceptible form.

Considerable work has been done on the relation of bacteriophage to dissociation. In 1921, Gratia obtained S and R type colonies from a single-cell strain of a sensitive coli culture. He found the R form considerably more resistant to the bacteriophage than was the original S culture. McKinley (1925) has reported the fact that forms of *B. coli* susceptible to bacteriophage differ from the resistant forms in the type of colony they produce, in that the susceptible colonies are smooth, whereas the resistant ones are rough. With both rough and smooth types, he has produced corresponding antiserums, which are type specific, and the antiserum for the smooth type also possesses an antilysin for the bacteriophage. Since there was no previous indication of the presence of bacteriophage in the pure culture of the smooth type, he has taken this to indicate (personal communication) that the organism possesses an inherent ability to produce bacteriophage. McKinley has made no mention of the changes in morphology which it is known may or may not occur in conjunction with the change from one type of colony to another.

Hadley (1927) reports that by the addition of either anti-S or anti-R lytic filtrate to S and R cultures of Shiga dysentery, marked lysis of the S culture is observed, whereas the R culture is but slightly affected. Likewise, Fejgin (1923) has found that certain R types of Shiga dysentery bacillus are resistant to the action of bacteriophage.

Hadley (1927), and Dawson (1933), have shown that the change

from S to R-type colonies is gradual, and that an organism may therefore produce colonies of varying degrees of roughness and smoothness. For this reason, the isolation of a completely susceptible form by a study of the morphology of the organism itself seems more profitable to us. No reference has been found in the literature to the effect that strains susceptible to bacteriophage have ever been obtained by single-cell isolations of definite morphological types.

In the case of the RB-2T strain, we have found the susceptible form to be coccoidal. That these coccoid forms do appear in cultures of *C. diphtheriae* has been recognized by Park and Williams (1929) and by other authors. The important work in this field has been summarized recently by Maver (1931), and by Pope and Pinfield (1932). This coccoid form is atoxic, producing in guinea pigs a severe lesion, which tends to remain localized, though in a few cases death occurs. The strain from which it was isolated, however, had been obtained from a severe case of diphtheria, and 1 cc. of the original twenty-four hour broth culture killed a 250-gram guinea pig in from one to four days. Lf's of the toxin produced *in vitro* by the method of Hazen and Heller (1931) ranged from 2.0 to 5.0. Further studies on the virulence and toxigenicity of these forms are in progress.

Of the other three partially susceptible strains that were obtained, one was slightly virulent as evidenced by guinea pig inoculation, one was completely avirulent although originally isolated from a typical case of diphtheria, and the third was a slightly virulent form of the P. W. No. 8 strain. As far as we know, a bacteriophage active against any form of the P. W. No. 8 strain has not been obtained previously. The organisms of these three partially susceptible strains were short, broad rods, usually less striated than the organisms of the typical No. 8 strain. Now and then, the two slightly virulent and invariably the avirulent one stained uniformly, with no striations.

The degree of susceptibility of *C. diphtheriae* to bacteriophage seems to increase as the organisms approach a coccoid form, and it is therefore for this less toxic form that the bacteriophage is most active.

CONCLUSIONS

1. Eighteen races of bacteriophage active against four strains of *C. diphtheriae* have been obtained: (a) from the intestinal contents of typical cases of diphtheria, (b) from the intestinal contents and peritoneal washings of guinea pigs injected with *C. diphtheriae*, (c) from cultures that have stood at room temperature four to six weeks.

2. Three partially susceptible strains of *C. diphtheriae* have been obtained, one of which is a No. 8 strain of partially reduced toxigenicity.

3. The titer of the bacteriophage has been increased by inoculation of 1 cc. of a weak race of bacteriophage plus 4 cc. of an eighteen hour culture of the homologous, partially susceptible organism into 100 cc. of broth, followed by incubation at 37°C. for two to four weeks.

4. A fourth completely susceptible strain has been obtained by isolation of a single cell coccoid culture from a diphtheria throat culture (RB-2T).

5. Using a completely susceptible culture (RB-2T single cell coccoid form), the titer of one race of bacteriophage has been increased to 10^{-6} by serial transfers of the susceptible culture with the lytic filtrate.

6. It has been shown that the coccoidal form of *C. diphtheriae* is the one stage in its development that is specifically susceptible to bacteriophage.

7. A description of the type of colony produced by this susceptible form (RB-2T single cell coccus) is given, and mention of its pathogenicity for guinea pigs is made.

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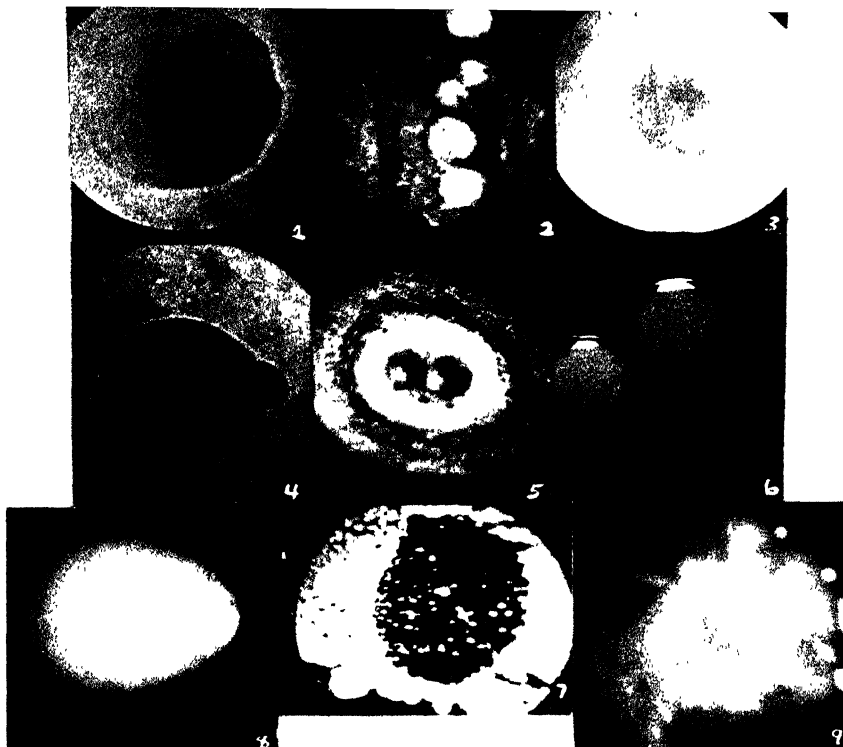


FIG. 1. 1. Park-Williams No. 8 grown on blood agar twenty-four hours 37°C. Smooth colony. $\times 100$.

2. P. W. No. 8 grown on blood agar twenty-four hours 37°C. Non-hemolytic smooth colony. $\times 100$ (From a water color drawing).

3. P. W. No. 8 grown on blood agar twenty-four hours 37°C. Mucoid colony. $\times 100$.

4. *C. diphtheriae* RB 2T throat culture grown on blood agar twenty-four hours 37°C. Smooth colony. $\times 200$.

5. *C. diphtheriae* RB 2T throat culture grown on blood agar twenty-four hours 37°C. Mucoid colony with double zone of hemolysis. Susceptible to bacteriophage. $\times 14$ (From water color drawing).

6. *C. diphtheriae* RB 2T single cell coccoid culture grown on blood agar twenty-four hours at 37°C. Mucoid colony. Susceptible to bacteriophage. $\times 28$.

7. *C. diphtheriae* RB 2T single cell coccoid culture with homologous bacteriophage. Incubated sixteen hours at 37°C. Veal-peptone agar. $\times 2.1$.

8. *C. diphtheriae*: RB 2T throat culture grown on blood agar forty-eight hours at 37°C. Mucoid colony. $\times 11.2$.

9. *C. diphtheriae*: RB 2T throat culture grown on blood agar ninety-six hours at 37°C. Mucoid colony. $\times 11.2$.

The photographs of the colonies were taken with a Leitz photomicrographic camera, using a Periplan 12 \times ocular. For figure 1, 1, 2, 4, 5, a Bausch and Lomb binocular microscope was used with reflected light from a Mazda 165-watt bulb. Figure 1, 3, 6, 7, was taken with a Bausch and Lomb AKW-5 widefield binocular with direct light from a Mazda 100-watt frosted bulb.

The organisms were photographed with a camera designed and constructed by Dr. C. B. Coulter and Dr. T. Rosebury of this department. A Leitz microscope with a 95 \times (1.32 mm.) fluorite oil immersion objective and a 12 \times ocular was used with a bellows extension on the camera giving about one-half times greater magnification. The source of light was a carbon arc lamp in combination with a Wratten G (No. 15) filter.



FIG. 2. 1. Park-Williams No. 8. Grown alternately on blood agar and on veal peptone broth. Twenty-four hour broth culture (37°C). Stained with alkaline methylene blue. $\times 1713$.

2. Park-Williams No. 8. Single cell coccoid culture grown on veal-peptone broth twenty-four hours (37°C). Stained with alkaline methylene blue. $\times 1713$.

3. Park-Williams No. 8. Single cell coccoid culture developing into rods; thirty-third transplant since isolation of single cell; twenty-four hour culture on veal-peptone broth (37°C). Stained with alkaline methylene blue. $\times 1713$.

4. Same as 3. Another stage.

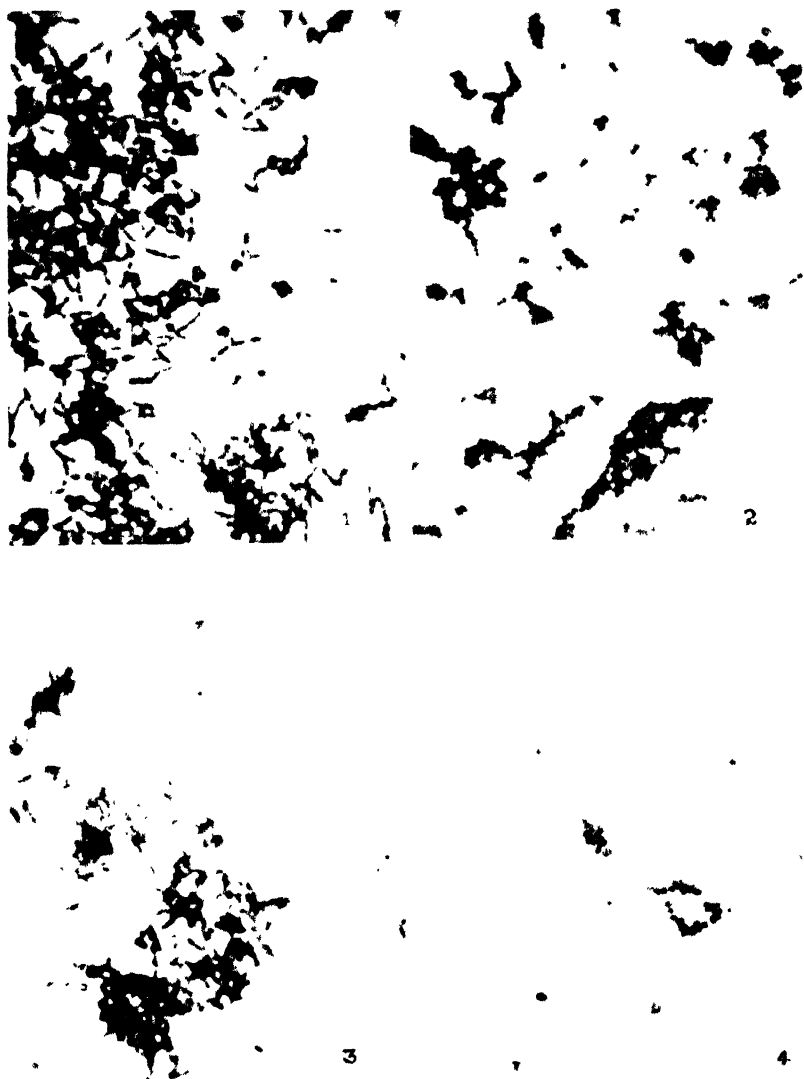


FIG. 3. 1. *C. diphtheriae*, RB 2T throat culture grown on veal-peptone broth twenty-four hours at 37° C. Stained with alkaline methylene blue. $\times 1713$.
 2. *C. diphtheriae*, RB 2T single cell coccoid culture grown on veal-peptone broth twenty-four hours at 37° C. Susceptible to bacteriophage. Stained with alkaline methylene blue. $\times 1713$.
 3. *C. diphtheriae*, RB 2T single cell coccoid culture developing into rods. Forty-third transplant since isolation of single cell. Twenty-four hour culture on veal-peptone broth (37° C.). Stained with alkaline methylene blue. $\times 1713$.
 4. *C. diphtheriae*, Organisms observed after growth of RB 2T single cell coccoid culture with homologous bacteriophage. Veal-peptone broth sixteen hours at 37° C. Stained with alkaline methylene blue. $\times 1713$.

THE EFFICIENCY OF CHLORINE IN SEWAGE DISINFECTION AS AFFECTED BY CERTAIN ENVIRONMENTAL FACTORS¹

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INTRODUCTION

Although sewage chlorination has been practiced for a number of years, there are still many factors affecting the efficiency of sewage chlorination which are poorly understood or practically unknown. It has been held that in order to produce bacterial removal in sewage a certain definite quantity of free chlorine must be present. Statements are made in the literature that chlorine kills bacteria present in sewage, immediately, when the chlorine is added, and also statements that the chlorine demand of the sewage caused by materials in solution and suspension must be satisfied before the bacteria are killed. The question of which substances interfere, if any, with the destruction of bacteria, whether some of the chloro-products formed are toxic, how these factors affect the time of contact of chlorine with sewage and the effect of varying dosages of chlorine, are all of theoretical and practical interest. It is presumably possible that with more knowledge the amounts of chlorine applied could be reduced, the construction of dosing devices and detention tanks altered and different points of application utilized which would be more effective and more practical.

With these general purposes in mind, the series of experiments reported and discussed below were planned to give more definite information with regard to the following aspects of the behavior

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of chlorine in sewage: (1) The effect of chlorine-dosage variation upon bacterial kill; (2) the effect of contact-time variation upon bacterial kill; and (3) an attempt to explain what happens to the chlorine which is absorbed in the sewage and what its ultimate effect is on the sewage constituents.

PROCEDURE

In an effort to determine the effect of dosage and contact-time on sewage chlorination, the 10-minute chlorine demand of the freshly collected, cotton-filtered sewage was determined by the orthotolidin test. Sewage was obtained from Plainfield, N. J., at the treatment plant after passing through fine screens. This sewage is considered domestic and stale. Sewage was also collected from New Brunswick, N. J., sewers, which contained trade waste and was not over one hour old. A residual of 0.2 p.p.m. was considered as the complete chlorine demand of the sewage. Dosages were then so arranged as to give 10, 20, 30, 40, 50, 60, 70, 90 and 100 per cent of this chlorine demand. The number of *B. coli* surviving were obtained by the presumptive test when contact periods of $2\frac{1}{2}$, 5, $7\frac{1}{2}$ and 10 minutes were employed. The contact periods were terminated by the addition of sodium thiosulfate. The second method of *B. coli* enumeration was by the use of ferrocyanide-citrate agar medium as devised by Tonney and Noble (1931). In the employment of this medium the contact periods used were $2\frac{1}{2}$, 5, $7\frac{1}{2}$, 10, 15, 20, 25 and 30 minutes. Total bacteria were determined by nutrient agar plates incubated at 20°C. for 48 hours, after the sewage was subjected for $2\frac{1}{2}$, 5, $7\frac{1}{2}$, 10, 15, 20, 25 and 30 minutes to chlorine dosages which represented 20, 40, 60, 80 and 100 per cent of the chlorine demand. When contact periods greater than 10 minutes were employed, the maximum chlorine dosages applied were 20 and 40 per cent respectively. The determinations in this case consisted of *B. coli* counts made on Noble's medium and total bacteria on nutrient agar incubated at 20°C. for 48 hours.

EFFECT OF CONTACT TIME AND CHLORINE DOSAGE

The effect of contact time and chlorine dosage on the per cent *B. coli* and 20°C. bacteria reductions is presented in tables 1, 2 and

3. It is apparent that a continuous reduction in the percentage of *B. coli* and 20°C. bacteria occurred with increasing satisfaction

TABLE 1

Effect of contact time and chlorine dosage on the per cent B. coli reduction (presumptive lactose broth medium)

Cl ₂ DEMAND	2½ MINUTES	5 MINUTES	7½ MINUTES	10 MINUTES
<i>per cent</i>				
0	0	0	0	0
10	0	22 1	31 7	40 3
20	48 1	58 3	62 7	47 2
30	51 0	68 8	79 2	74 0
40	66 9	80 5	84 0	82 0
50	51 0	83 0	89 7	87 7
60	75 2		86 8	93 0
70	89 7		95 9	99 3
90	95 9	99 5	98 9	99 9
100	97 3	99 6	99 9	99 9

Note. Each result is the average of four experiments.

TABLE 2

Effect of contact time and chlorine dosage on the per cent B. coli reduction (citrate agar medium)

Cl ₂ DEMAND	2½ MINUTES	5 MINUTES	7½ MINUTES	10 MINUTES	15 MINUTES	20 MINUTES	25 MINUTES	30 MINUTES
<i>per cent</i>								
0	0	0	0	0	0	0	0	0
10	5 3	9 8	16.5	16 7	65 5	61 5	66 4	76 7
20	24 6	19 8	26.7	44 0	78 8	83 4	84 1	97 2
30	37 0	45 6	44 2	49 4				
40	45 4	69 8	58 6	63 0				
50	64 5	77 3	80 1	72 5				
60	84 0	75 2	83 5	83 6				
70	90 1	78 5	93.1	97 5				
90	96 6	88 4	98 0	99 6				
100	98 2	92.9	99 1	98 6				

Note: The per cent *B. coli* reductions obtained with 2½, 5, 7½ and 10 minute contact periods are the average of four experiments, whereas the reductions obtained with 15, 20, 25 and 30 minute contact periods are the averages of 8 and 9 experiments.

of the 10-minute chlorine demand of the sewage. As much as 90 per cent or more *B. coli* reduction was obtained when 70 per cent

of the 10 minute chlorine demand was satisfied at contact periods of $2\frac{1}{2}$, $7\frac{1}{2}$ and 10 minutes. The dilution method of computing *B. coli* numbers inevitably gives large fluctuations. With lower chlorine-demand satisfaction the changes in contact time caused greater variations in the kill of *B. coli*.

Table 2 shows that the per cent *B. coli* reduction is greater for each 10 per cent increase in the satisfaction of the chlorine demand. Here again (with the use of Noble's medium) a *B. coli* reduction of over 90 per cent could be produced when 70 per cent of the chlorine demand was satisfied and contact periods of $2\frac{1}{2}$, $7\frac{1}{2}$

TABLE 3

Effect of contact time and chlorine dosage on the per cent 20°C. bacteria reduction (nutrient agar medium)

Cl ₂ DEMAND	2½ MINUTES	5 MINUTES	7½ MINUTES	10 MINUTES	15 MINUTES	20 MINUTES	25 MINUTES	30 MINUTE
<i>per cent</i>								
0	0	0	0	0	0	0	0	0
20	16.6	32.5	39.1	43.0	56.8	70.3	80.9	86.8
40	37.6	57.8	69.5	90.0	94.3	98.3	99.4	99.8
60	56.3	84.5	96.9	96.9				
80	75.7	94.8	96.6	99.5				
100	89.9	95.6	98.2	99.8				

Note: The per cent reductions obtained with $2\frac{1}{2}$, 5, $7\frac{1}{2}$ and 10 minutes are the average of three experiments, whereas the reductions obtained with 15, 20, 25 and 30 minutes are the averages of 2 experiments.

and 10 minutes were maintained. The average of the contact periods for 70 per cent chlorine-demand satisfaction show a reduction of 89.8 per cent, while the next 30 per cent satisfaction caused an additional average reduction of 9.8 per cent *B. coli*.

Variations in the contact period and the per cent additions of the chlorine demand produce variations in the per cent reduction of the total bacteria. As the reaction time increases, the number of bacteria surviving chlorination decreases. Contact periods affect not only the extent to which bacteria are killed, but also the per cent to which the chlorine demand needs be satisfied. The relation between chlorine-demand satisfaction and contact time as affecting bacterial reduction is clearly brought out in figure 1.

With progressive increases of the chlorine-demand satisfaction bacterial reduction becomes greater but not in direct proportion. With increasing contact time and equal chlorine-demand satisfaction the kill increases, no matter whether the chlorine demand satisfaction is of the order of 20, 40, 60, 80 or 100 per cent.

The relation between reaction time and the per cent *B. coli* reductions obtained by the use of ferrocyanide-citrate agar medium is shown in figure 2, where results obtained with additions of 10 and 20 per cent of the chlorine demand are plotted. Figure 3 presents similar relations between reaction time and the per cent

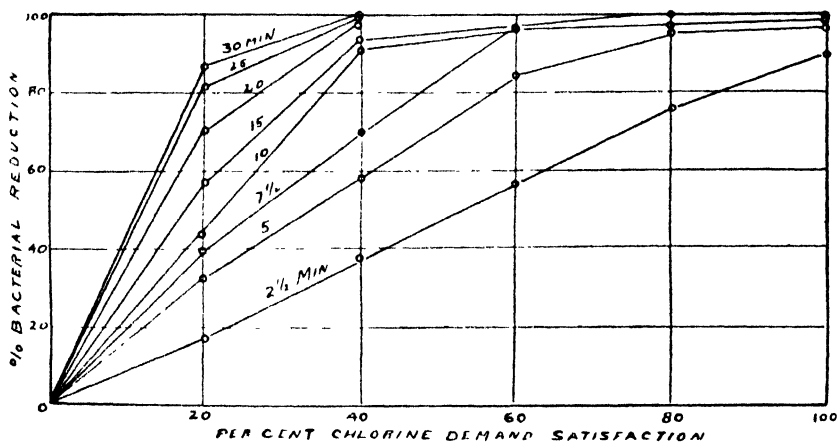


FIG. 1. EFFECT OF CONTACT TIME AND CHLORINE DOSAGE ON 20°C. BACTERIA REDUCTION

20°C. bacterial reductions as obtained by the nutrient agar medium, but with 20, 40 and 60 per cent demand satisfaction. From the curves in these figures two general conclusions may be drawn: (1) The reaction time between chlorine and sewage affects the extent to which the bacteria are killed. The longer the reaction period, the greater the per cent reduction in bacteria. (2) The amount of chlorine added in respect to the chlorine demand likewise affects the extent to which the bacteria are reduced. The greater the per cent chlorine-demand satisfaction, the greater the per cent reduction in the bacteria. For a given bacterial reduction, the greater the amount of chlorine added, expressed as

a percentage of the chlorine demand, the shorter will be the contact period required.

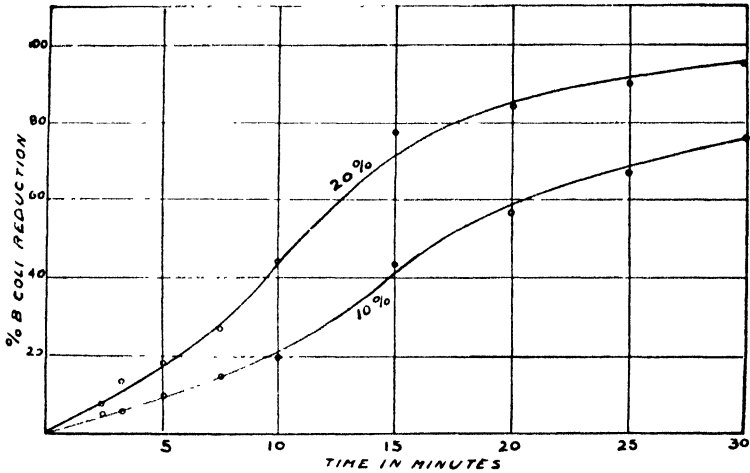


FIG. 2. RELATION BETWEEN B. COLI REDUCTION, CHLORINE DEMAND SATISFACTION AND CONTACT TIME

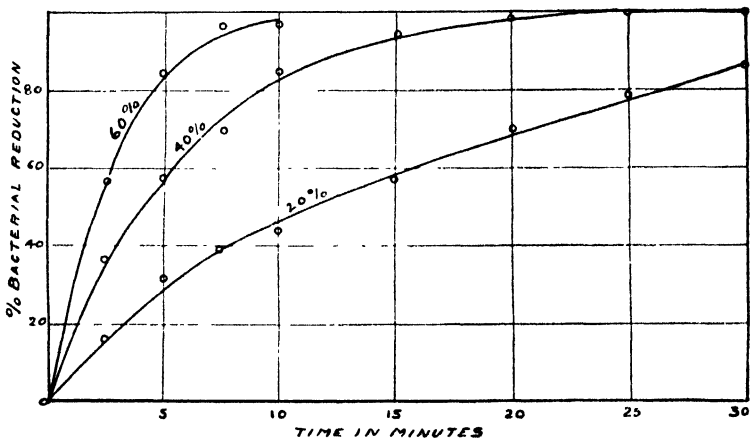


FIG. 3. RELATION BETWEEN 20°C. BACTERIA REDUCTION, CHLORINE DEMAND SATISFACTION AND CONTACT TIME

It is evident from the results that statements in the literature which imply that complete satisfaction of the chlorine demand is

necessary before bacterial destruction takes place, are incorrect. Long before all chlorine demand is satisfied bacteria are killed. As a matter of fact the percentage kill is materially greater than the percentage chlorine satisfaction when contact periods of 5 minutes or more are employed. The story may be different when the largest part of the chlorine demand is caused by materials in solution, especially gases and organic substances in a highly putrescible state. For instance, when septic tank effluents are chlorinated, which contain relatively large quantities of H_2S and soluble organic intermediate decomposition products, none or very little kill is obtained unless an excess of chlorine is applied. Our results show also that, with the addition of small quantities of normal urine, at least 50 per cent of the chlorine demand must be satisfied before kill occurs with a contact period of 15 minutes.

It has been held by some investigators that chlorine, when applied to sewage, would have a selective action. The chlorine is assumed to satisfy substances in solution, organic material and bacteria in the order given. That this is not exactly correct is shown by the results presented above. The bacterial death rate is high for a low per cent chlorine-demand satisfaction. This high per cent bacterial kill indicates that the reaction is not selective. It appears that bacterial reduction takes place simultaneously with the reaction of chlorine with soluble substances.

There may be three general reactions in the mechanism of chlorinating below the residual: (1) The chlorine combines with existing oxidizable materials to form compounds which are deficient in germicidal properties. (2) A chloro-substitution reaction occurs, resulting in the formation of chloro-compounds (chloro-amino acids, chloro-organic acids and chloramines) which may or may not be possessed of toxic properties. These chloro-compounds fix or loosely bind the chlorine. (3) With short contact periods, the chlorine may directly react with the bacteria. As the contact period is increased, bacterial reductions are brought about by the chloro-products as well as by the primary chlorine. With still greater contact periods the disinfection reaction may be increasingly affected by chloro products. The longer the contact time the greater will be the amount of chloro-

organic products possessing a low oxidation potential, provided there is chlorine available for the substitution reaction.

When chlorinating to less than residual, it is important to know for what period the chlorine and sewage have been in contact. Low percentages of chlorine-demand satisfactions require a longer period to accomplish a definite bacterial reduction than do higher percentages of chlorine-demand satisfaction. When the chlorine demand is completely satisfied, 20°C. bacteria reduction takes place rapidly. Table 3 shows that reductions of 89 and 95 per cent are obtained with contact times of 2½ and 5 minutes respectively, but 10 minutes are required to produce a 99.8 per cent reduction. *B. coli* reduction appears to be more rapid than total bacteria reduction.

The absorption of chlorine is very rapid. It is well known that the resulting bacterial removal is controlled to a great extent by the homogeneous and immediate admixture of chlorine and sewage. Our results show that there is a definite relation between the amount of chlorine added and the kill obtained even though there is no residual chlorine present. It should be kept in mind that in all our trials the chlorine was well mixed with the sewage. Inefficient mixing leads to local concentration of chlorine, which results in loss of effectiveness of the disinfectant. Contradictory results obtained and reported from different plants may be attributed in large part to the method and points of chlorine application. When the point of chlorination is improperly selected poor results may be expected. Some of the advantages of pre-chlorination or split chlorination may be attributed to the better mixing, as well as the longer time of contact. Application of chlorine in a sewer will produce greater bacterial kill than the same amount of chlorine added in a contact tank where the mixing is relatively slower and often incomplete. Properly constructed diffusers produce a more homogeneous sewage-chlorine mixture and of necessity produce greater and more rapid kill.

The results obtained indicate that a 90 or greater per cent 20°C. bacterial removal is assured with detention periods varying from 10 to 30 minutes when the sewage is chlorinated to the extent of but 40 per cent of the chlorine demand, but less when the contact

time is shorter. If partial chlorination is practiced at places where large volumes of diluting water are present, sufficient contact time must be given. Since chlorinating to the extent of 40 per cent of the chlorine demand results in the reduction of over 98 per cent of the 20°C. organisms when 20, 25 and 30 minute contact periods are maintained, it is possible, therefore, to chlorinate on the basis of a fixed dosage. Tiedeman (1927) at Huntington, L. I., found that the seasonal chlorine demand, based on the maximum daily demands during 1926, varied from 6.5 to 13 p.p.m. with a yearly average of 9.6 p.p.m. The increased efficiency to be affected by chlorinating for 15 minutes or more with 40 per cent of the yearly average chlorine demand is apparent when chlorination is properly controlled. On the basis of the yearly average chlorine demand only 3.84 p.p.m. chlorine would be required to effect a 98 per cent reduction of bacterial counts, provided an intimate contact between the chlorine and sewage could be maintained for 15 to 30 minutes. Results reported and discussed elsewhere in this paper tend to show that the chlorine dosage does not have to be applied necessarily in proportion to chlorine-demand variation, because the quantity of chlorine is not dependent upon the total chlorine demand of fresh sewage, but upon those substances which have a great avidity for chlorine. These substances increase with the age of the sewage.

EFFECT OF INTERFERING SUBSTANCES

a. Lactose, peptone, ammonia and urine

The types and numbers of possible substances interfering with the kill of bacteria when liquid chlorine is added to sewage presumably vary with the character and type of sewage used. It was impossible to try a very large number of materials and it was decided to pay particular attention to materials always present in comparatively large quantities and also to materials which would represent certain classes of materials. It is well known that amino acids have an avidity for chlorine and that gases such as H_2S react readily with chlorine. Since ammonia and urine or urea are always present, experiments were made with additions of $(\text{NH}_4)_2\text{CO}_3$, NH_4HCO_3 and different quantities of normal urine.

As a representative of carbonaceous material the effect of lactose was studied, and as a representative of a highly complex molecule containing nitrogen, peptone was selected.

In these experiments, 10 and 50 p.p.m., of peptone, lactose, $(\text{NH}_4)_2\text{CO}_3$ and NH_4HCO_3 and 1 cc. and 5 cc. of urine solution were added to 100 cc. of freshly collected cotton-filtered sewage. An amount of chlorine equal to one-half the chlorine demand was added and left in contact with the sewage for 10 minutes.

The figures in table 4 show that additions up to as much as 50 p.p.m. of lactose, peptone and fixed ammonia solutions did not have an effect on bacterial reductions. The variations are within the experimental error with the high dilutions employed. When 5 cc. of normal urine solution was added chlorination was interfered with remarkably. The chlorine demand of the sewage plus the above mentioned materials (with the exception of urine) by the starch-iodide tests were as follows:

	p p m.
Sewage.....	12 32
Sewage and 50 p.p.m. peptone.	12 32
Sewage and 50 p.p.m. $(\text{NH}_4)_2\text{CO}_3$	12.32

b. Chloro-peptones and chloro-protein

a. Chlorination of nutrient broth with varying amounts of chlorine and inoculation with as much as 1 cc. of sewage.

In an attempt to determine the effect of chloro-proteins and chloro-peptones on bacteria, 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 p.p.m. of chlorine were added to a 0.5 per cent solution of peptone in nutrient broth. The contact period employed between the chlorine and nutrient broth was 30 minutes. One cubic centimeter and 2 platinum loopfuls of sewage were then added to individual tubes containing the varied amounts of chloro-peptones and chloro-proteins. The tubes were incubated at 37°C . for 24 hours, examined for growth and confirmed by streaking upon agar slants.

As an illustration, the results of two experiments are given in table 5. There was no inhibitory effect upon the growth of sewage organisms when as little as 0.1 p.p.m. or as much as 5.0

p.p.m. chlorine were added to nutrient broth solution. In this case the toxicity of the chloro-nitrogenous substances was not of sufficient strength nor amount to indicate growth inhibition.

b. Chlorination of nutrient broth to various percentages of the one-hour chlorine demand and its effect upon an inoculation of a *B. coli* suspension.

This experiment was made for the purpose of determining to what extent nutrient broth must be chlorinated in order to inhibit the growth of bacteria. Varying percentages of chlorine, ex-

TABLE 4

Effect of addition of peptone, lactose, ammonia and urine solutions to sewage upon the per cent reduction of 20°C. organisms (contact time 10 minutes)

	PER CENT Cl_2 DEMAND						
	0			50			Average
	3 90	5 75	12 32	3 90	5.75	12 32	
Cl_2 demand, p.p.m.							
Control	0	0	0	45 3	96 3	97.0	79 5
Peptone, 10 p p m.	0	0	0	40 6	94.7		
Peptone, 50 p.p.m.	0	0	0	33 3	91.3	95 6	73 4
Lactose, 10 p.p.m.	0	0	0	41 6	96 6		
Lactose, 50 p.p.m.	0	0	0	40 0	95 1	98 0	77.7
$(\text{NH}_4)_2\text{CO}_3$, 10 p.p.m.	0	0	0	41 3	95 4		
$(\text{NH}_4)_2\text{CO}_3$, 50 p.p.m.	0	0	0	38 0	94 7	98 5	77.0
NH_4HCO_3 , 10 p.p.m.	0	0	0				
NH_4HCO_3 , 50 p.p.m.	0	0	0	41 0	94 0	97.2	77 4
Urine, 1 cc	0	0	0	41 0	61 8	46 2	48 8
Urine, 5 cc	0	0	0	0		4 8	2 4

Note: Each result is the average of two trials.

pressed as percentages of the one-hour chlorine demand, were introduced into 10 cc. of nutrient broth. After standing for one hour, a loopful of a *B. coli* suspension was added to each fractionally chlorinated nutrient broth solution. The tubes containing the various admixtures were incubated for 24 hours at 37°C. The presence of *B. coli* was then confirmed by inoculating 1 cc. of each fractionally chlorinated nutrient broth into lactose broth fermentation tubes.

From the results in table 6 it is evident that the growth of *B. coli* is inhibited when 80 per cent of the one-hour chlorine demand of nutrient broth is satisfied.

The one-hour chlorine demand of the nutrient broth solution by the starch acid-iodide test was 179 p.p.m.

TABLE 5
*Effect of chloro-peptones on B. coli growth**

Cl ₂ p.p.m.	Cl ₂ RESIDUE AFTER 24 HOURS	GROWTH	CONFIRMED BY AGAR PLANT
0	—	+	+
0.1	—	+	+
0.2	—	+	+
0.4	—	+	+
0.6	—	+	+
0.8	—	+	+
1.0	—	+	+
1.5	—	+	+
2.0	—	+	+
3.0	—	+	+
4.0	—	+	+
5.0	—	+	+

* These results were confirmed on two different occasions.

TABLE 6
Effect of increasing per cent chlorine demand satisfaction of nutrient broth upon the growth of B. coli

Cl ₂ DEMAND per cent	GROWTH	CONFIRMED BY LACTOSE BROTH	
0	+	+	+
20	+	+	+
40	+	+	+
60	+	+	+
80	—	—	—
100	—	—	—

c. The effect of peptone solutions chlorinated to different percentages of the chlorine demand upon equal volumes of sewage.

The question arose whether addition of varying percentages of chlorine to peptone solution expressed as percentages of the chlorine demand, had any effect on the killing of sewage bacteria. It was thought that more consistent results in the bacterial kill would be obtained by the direct nutrient agar plate method.

The amount of chlorine necessary to satisfy the one-hour chlorine demand of a 0.5 per cent peptone solution was 7.64 p.p.m. A one-hour contact period was maintained with the peptone solution and with 0, 20, 40, 60, 80 and 100 per cent of its one-hour chlorine demand additions. Of these, 50 cc. fractionally chlorinated peptone solutions were left standing for one hour with 50 cc. of raw sewage. The numbers of 20°C. bacteria were determined on nutrient agar after 48 hours of incubation. The controls consisted of equal volume mixtures of sewage and buffered distilled water. Table 7 indicates that no matter to what extent a 0.5 per cent peptone solution is chlorinated in respect to its one-hour chlorine

TABLE 7

Effect of increasing per cent chlorine demand satisfaction of a 50 per cent peptone solution upon an equal volume of sewage

Cl ₂ DEMAND	BACTERIA PER CUBIC CENTIMETER	REDUCTION
<i>per cent</i>		<i>per cent</i>
0	3,195,000	0
0 (1 to 1)	430,000	86 5
20 (1 to 1)	425,000	86 7
40 (1 to 1)	925,000	74 1
60 (1 to 1)	455,000	85 7
80 (1 to 1)	393,000	87.7
100 (1 to 1)	431,500	86.5

demand, the reductions obtained agree closely with those obtained upon the addition of 50 cc. of sewage to 50 cc. of buffered distilled water. The results obtained still further show that chloro-peptones have for all practical purposes no germicidal properties.

Recent research work on the relationship between chemical constitution and antibacterial action of phenol derivatives seem to point generally to the importance of size of the molecule as one of the major factors influencing the degree of bactericidal efficacy. By analogy it is quite possible that similar relationships would exist with chloro-products (see Klarman, etc., 1933).

c. The effect of adding one part of chlorinated sewage to one and four parts of untreated sewage.

Five hundred cubic centimeters of fresh unfiltered sewage were

chlorinated to complete chlorine-demand satisfaction after determining the 10-minute chlorine demand by the acid starch-iodide test. The sewage and chlorine were left standing for one hour. The number of 20°C. bacteria was then determined. One part of the chlorinated sewage was added to one and four parts of untreated sewage. These mixtures were allowed a contact time of one hour after which the numbers of 20°C. bacteria were determined. The results are shown in table 8.

When the chlorine demand of a 100 cc. sample of sewage was satisfied and the sample left standing for one hour, no residual chlorine was indicated by the acid starch-iodide test. And when 50 cc. of this chlorinated sewage containing no available

TABLE 8
Effect of addition of chlorinated sewage to untreated sewage

MIXTURES	BACTERIA IN THOUSANDS PER CUBIC CENTIMETER	BACTERIAL REDUCTION
		<i>per cent</i>
Unchlorinated sewage	8450	
One part treated and 1 part untreated sewage	2 5	99 96
Unchlorinated sewage	2415	
Chlorinated to 100 per cent Cl_2 of demand for 1 hour	6 8	99 7
One part treated and 1 part distilled water	1 2	99 99
One part treated and 1 part untreated sewage	14 5	95 3
One part treated and 4 parts untreated sewage	700	70.1

chlorine was left in contact with 50 cc. of untreated sewage for one hour, a 95 or greater per cent bacterial reduction was obtained. This indicates that chloro-products produced in sewage as well as available chlorine may bring about bacterial reductions.

Effective disinfection of sewage does not depend upon the persistence of primary chlorine (free chlorine, hypochlorites or hypochlorous acid). Sewage disinfection is affected by the germicidal chloro-organic products produced by virtue of reactions between chlorine and organic compounds. Bacterial reductions produced by chloro-products are very small when the contact time between chlorine and the sewage constituents is short, for the amounts of chloro-products formed are comparatively small. Increasing the

contact time results in the formation of more chloro-products and, consequently, in greater bacterial reductions. In this process the early bacterial reductions are produced by primary chlorine, but with longer reaction periods the bacterial reductions are caused to a greater extent by the chloro-products.

EFFECT OF CHLORINE-DEMAND VARIATIONS

A study of the effect of chlorine-demand variations upon bacterial removal was pursued in accordance with the following two procedures:

1. After the chlorine demand of the raw sewage was determined, it was diluted with distilled water in the following ratios: one

TABLE 9

Effect of chlorine demand variations upon per cent B. coli reduction (contact time 15 minutes)

TOTAL CHLORINE DEMAND	Cl ₂ DEMAND SATISFIED	BACTERIA PER CUBIC CENTIMETER	BACTERIAL REDUCTION
<i>p p m</i>	<i>per cent</i>		<i>per cent</i>
17 42	0	524,000	
	20	8,000	98 4
	40	350	99 9
8 71	0	90,000	
	20	6,100	93.2
	40	1,130	98 7
4 35	0	63,000	
	20	24,700	60 8
	40	2,260	96 4

part of sewage to one part of distilled water and one part of sewage to three parts of distilled water. The chlorine demand of the sewages diluted to 1:2 and 1:4 of its original concentration was determined to be 1:2 and 1:4 respectively that of the undiluted sewage. The sewages were then chlorinated to the extent of 10 and 20 per cent of the chlorine demand for 15 minutes. The number of *B. coli* surviving chlorination was determined by Noble's cyanide-citrate agar medium. Table 9 shows the results.

2. By treating sewage for 15, 20, 25 and 30 minutes with 10 and 20 per cent of the chlorine demand, it was possible to obtain

a percentage of *B. coli* reductions by Noble's medium which varied according to the chlorine demand variation of the sewage. The relationship between the per cent *B. coli* reduction and the chlorine demand variation is shown in figure 4.

A variation in the chlorine demand of the sewage affects the per cent *B. coli* reduction (table 9). Sewages with varied chlorine

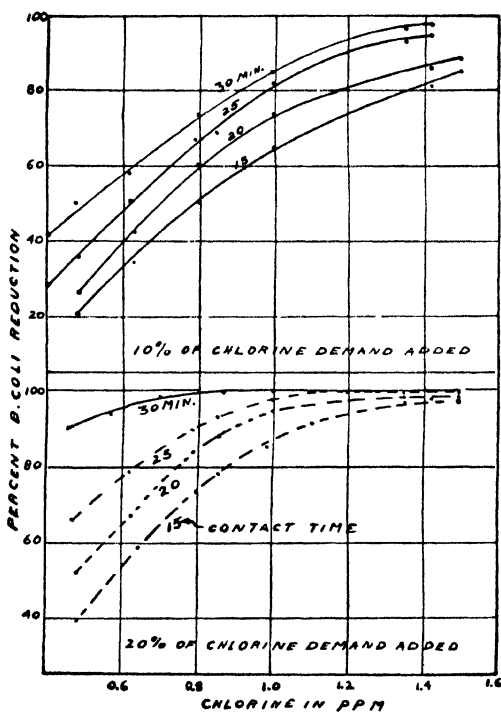


FIG. 4. RELATION BETWEEN *B. COLI* REDUCTION AND CHLORINE DEMAND VARIATION

demand chlorinated to the extent of 10 and 20 per cent of their chlorine demands show that those having a small chlorine demand affect a smaller per cent of *B. coli* removal than sewages having a large chlorine demand. The effect of the chlorine-demand variation is less pronounced when the chlorine demand is satisfied to the extent of 40 per cent.

EFFECT OF STORAGE

Sewage was collected and stored at 0° and 20°C. for a number of days. Bacterial numbers were determined from day to day. Portions of the sewage were chlorinated daily to 10 and 20 per cent of the chlorine demand and contact times maintained rang-

TABLE 10
Effect of storage at 0°C. and 20°C. on bacterial numbers

STORAGE DAYS	20°C		0°C.	
	B coli	Reduction per cent	B coli	Reduction per cent
0	72,000	0	72,000	0
1	69,000	4 1	37,000	47 7
3	56,000	22 2	26,000	63.8
7	31,000	56 9	30,000	58 3

TABLE 11

DAYS STORAGE	CONTACT TIME							
	15 min		20 min		25 min		30 min	
	Per cent Cl ₂ demand reduction							
	10	20	10	20	10	20	10	20

Storage at 20°C. and partial chlorination after different days

	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
0	54 1	77 9	66 6	84 6	67 1	90 3	72 0	93 5
1	66 6	93 9	59 5	98 4	75 2	99 5	86 5	
3			56 3	99 1	64 1	99 6	89 2	99.9
7	92 5	98 4	96 2	98 8			97 7	99 4

Storage at 0°C. and partial chlorination after different days

	54.1	77.9	66 6	84 6	67.1	90 3	72.0	93.5
0	54.1	77.9	66 6	84 6	67.1	90 3	72.0	93.5
1	80.5	97.8	88 1	98 6	88 2		93.7	
3	83 8	98 2	87.4		95.0		97.4	99.0
7			86.2	95 2	86.9	98 0	96 6	

ing from 15 to 30 minutes. The results of the storage without chlorination are given in table 10 and those obtained after chlorination in table 11.

Storage of sewage at low temperatures indicates a greater per cent *B. coli* reduction upon chlorinating after the first day's stor-

age than at higher temperature. Chlorination after one day or more storage shows a greater percentage reduction, both for 0°C. and 20°C. storage, than of the fresh sewage. Just what the cause may be for the greater per cent bacterial reductions is difficult to explain. There is no evidence pointing in the direction that the decomposition of the sewage is responsible. Decomposition of material at a low temperature is practically nil, but *B. coli* reduction after chlorinating the stored sewage at 10°C. was the same as in the sewage stored at 20°C. Houston's (1913) hypothesis of devitalization may be an explanation. Results obtained by Heukelekian in this laboratory show that there is a rapid increase in the number of *B. coli* up to 24 hours after storage. After 24 hours, the numbers of *B. coli* decrease very rapidly. It is possible that at the point when the numbers of *B. coli* decline rapidly the organisms become more sensitive to the toxicity of the applied chlorine so that a greater per cent are killed by chlorine following 24 hours storage. When a given reaction period is maintained, the per cent bacterial removals with low per cent chlorine demand satisfaction varies with the chlorine demand which in turn, affects the amount of chlorine to be added for the given per cent chlorine demand satisfaction (fig. 4). For instance, when a 15-minute contact period was maintained, higher bacterial reductions were produced when the amount of chlorine added in respect to a given per cent chlorine-demand satisfaction was large. For the same contact period, when the amount of chlorine added in respect to a given per cent chlorine demand satisfaction was small, the per cent bacterial reductions were low. Increasing the contact time provided for greater bacterial reductions while the low chlorine concentration affected the per cent bacterial removals as did the lower contact periods.

If there were no interfering substances, the per cent bacterial kill would be proportionate to the amount of chlorine added. The results do not give proportionate per cent bacterial removals upon increasing chlorine dosages. It is, therefore, evident that there are substances in the sewage which consume chlorine and hence take away the chlorine which should be available for disinfection purposes. The greater the demand for chlorine, the larger is the

amount of interfering substances. The larger the amount of interfering substances, the lower is the sterilization efficiency. However, the differences become less with the increase in contact time.

Sewage chlorination, when properly controlled, can be accomplished by chlorinating on the basis of a fixed dosage. Regardless of what the chlorine demand of the sewage may be, the results tend to show that the amount of chlorine added to sewage determines to what extent the bacteria are killed.

EFFECT OF DIALYSIS AND ELECTRO-OSMOSIS

In connection with the studies made to determine the nature of the substances interfering with the kill of the organisms, some

TABLE 12
Effect of dialysis upon the chlorine demand (in p.p.m.)

NUMBER	BEFORE DIALYSIS	AFTER 48 HOURS			AFTER 96 HOURS		
	Raw sewage	Raw sewage	Internal liquid	Dialysate	Raw sewage	Internal liquid	Dialysate
1	6 98			8 63			
2	4 66	4 96	12 06	7 71	5 46	13.19	11 01
3	5 50	5 85	13 24	13 24	6.31	14 01	10.78
Average.	5 08	5 40	12 65	10 47	5 88	13.61	10 89

trials were made to determine the effect of dialysis upon the chlorine demand of the liquid. Although the data obtained are not sufficient to permit the drawing of definite conclusions, they were thought of sufficient interest to include as an indication and possible guide for further studies.

Two groups of trials were made: dealing (1) with the dialysis of sewage through parchment paper, and (2) with electro-osmosis through a porous membrane.

1. Effect of dialysis

Table 12 shows the results obtained by dialyzing sewage for 48 and 96 hours. With the second and third series of experiments, the increase in the chlorine demand is 0.8 p.p.m. when the raw

sewage is permitted to stand for 96 hours at room temperature. Upon dialyzing for 48 hours the average chlorine demand of 5.08 p.p.m. of the raw sewage increased about two and one-half times or to 12.65 p.p.m. The average chlorine demand of the sewage material which passed through the parchment paper was, after 48 hours, double that of the raw sewage. The distilled water was then replaced and dialysis continued for another 48 hours. The sewage which was placed in the parchment paper had a chlorine demand of 13.6 p.p.m. or slightly less than 1.0 increase from the previous 48 hours of dialysis. The chlorine demand of the dialysate was nearly the same as the chlorine of the dialysate after the first 48 hours of dialysis. Since the volume of the sewage was the same as the distilled water used each time (1000 cc.) the total quantity of water was 2000 cc. or double the quantity of sewage. Letting the sewage stand at the same temperature (room) as the dialyzed material an increase of only 0.48 p.p.m. chlorine demand was obtained, whereas, the total chlorine demand of the dialyzed sewage and the dialysate amounted to 35.39 p.p.m. or an increase of 29.99 p.p.m.

2. *Effect of electro-osmosis*

Two trials were made by subjection of the sewage to an electric current for one hour. The results were as follows: Direct current 108 volts, 1.0 amperes; sewage used (internal fluid), 300 cc.; distilled water used (external fluid), 750 cc.; dialysis for one hour.

The chlorine demand determined by acid starch-iodide test and expressed in parts per million was as follows:

	<i>Trial 1</i>	<i>Trial 2</i>	<i>Average</i>
Raw sewage.....	21.49	21.49	21.49
Dialysate.....	8.25	8.35	8.30
Internal fluid.....	1.17	1.17	1.17

The amount of distilled water used was two and one-half times greater than the volume of raw sewage so that the chlorine demand of the total quantity of external fluid was 20.75 p.p.m. on an equal-volume basis. The chlorine demand of the sewage (internal fluid) together with the demand of the dialysate was 21.92 p.p.m. or only very slightly greater than the demand of the raw sewage.

These results would seem to be contradictory to those obtained with dialysis. It is well known that the chlorine demand of sewage increases upon standing, presumably due to bacterial action which causes hydrolysis or splitting up of more complex organic material to simpler compounds. When sewage was dialyzed the time required was 48 to 96 hours, sufficient to produce a quantity of liquified material with a high chlorine demand. On the other hand electro-osmosis was completed in one hour. From other work in our laboratory it is known that inorganic materials in addition to organic substances pass rapidly through the membrane when electro-osmosis is applied. Since several of the inorganic materials passing through the membrane have practically no chlorine demand it seems clear that the organic substances passing through the membrane are chiefly responsible. These organic substances appear to be in solution but can be readily flocculated. The results show that the chlorine demand of the total mass did not materially increase, whereas the chlorine demand of sewage left standing increased materially. The same was the case with dialyzed sludge, the process in the case requiring from two to four days. During this time bacterial action was sufficient to produce an additional quantity of liquified compounds, which required an additional amount of chlorine to be satisfied. The materials which are in the transition state (changing from complex to simpler compounds) are therefore, the interfering substances. This reasoning leads us to the dictum that the fresher the sewage, the less chlorine should be required for an equal kill of bacteria. We find however, that upon storage the kill is increased. Obviously therefore, there are two factors to be taken into consideration: (1) quantity of interfering substances produced (materials with high chlorine demand) and (2) resistance of bacteria. The results indicate that, although more chlorine is required for the satisfaction of liquified products, less chlorine is necessary to kill the surviving bacteria after storage. It is therefore, a question which of the two factors is predominant. If interfering substances are produced at a relative rapid rate the kill will be less, with equal quantities of chlorine, than if the interfering substances are produced at a slow rate. This has been shown in

actual practice (difference between summer and winter) and in the laboratory experiments with sewage stored at 0° and 20°C. If, on the other hand, the resistance of the bacteria surviving storage decreases at a greater rate than the increase in interfering substances, less chlorine would be necessary in spite of the fact that the sewage has a greater chlorine demand. The results reported above showing a greater percentage bacterial reduction on the stored sewage as compared with the fresh sewage would be an example.

The results obtained by electro-osmosis indicate also that the chlorine demand exerted by the particles in suspension is slight, which in turn would indicate that oxidation of such material by chlorine is of little importance. Any reduction in oxygen demand of sewage caused by chlorine would be limited (for practical purposes) to the organic material in solution. Some investigators advance the theory (Jenkins 1931-32) that accumulation in solution and at the surface of the sludge particles of substances which raise a physical barrier between the organisms and the solid particles will retard decomposition. This could also be applied in a more direct chemical sense, whereby the chlorine is prevented from attacking the bacteria which are surrounded with materials in solution having a strong avidity for chlorine. The inorganic materials would not interfere directly by reacting with the chlorine applied, but may form a physical barrier to the chlorine so that organisms can not be reached unless a longer contact time is available or a more perfect distribution of the chlorine is obtained.

As stated above the data available are insufficient to draw definite conclusions in regard to the specific materials which interfere with the kill, but they indicate that they are organic materials in solution rather than inorganic salts or the coarser suspended solids. The results indicate also a fruitful field for further investigations.

SUMMARY AND CONCLUSIONS

Studies were made on the effect of increasing per cent chlorine-demand satisfactions upon *B. coli* and 20°C. bacterial reductions; the effect of contact periods varying from 2½ to 30 minutes upon

bacterial reductions; the effect of storage upon chlorination; the effect of chloro-proteins and chloro-peptones upon bacterial reductions, the effect of the addition of peptone, lactose, fixed ammonia solutions and urine upon bacterial reductions after chlorination; the effect of chlorine-demand variations upon bacterial reductions and the effect of the addition of chlorinated sewage to untreated sewage. Some preliminary work was done on determining the substances which have greatest affinity for chlorine by dialyzing raw sewage.

From these studies the following conclusions may be drawn:

1. Bacterial reductions are obtained with partial chlorination of sewage. The reductions vary with the per cent chlorine-demand satisfaction.

2. The per cent bacterial reductions produced appear to be affected not only by the per cent chlorine-demand satisfaction, but also by the amount of chlorine required to accomplish the given per cent satisfaction.

3. The rate of bacterial kill is greater with lower chlorine-demand satisfactions.

4. Contact period variations affect the per cent bacterial reductions when the sewage is chlorinated below the residual.

5. Contact periods greater than $2\frac{1}{2}$ minutes do not appear to affect greatly the per cent bacterial reductions when the chlorine demand is satisfied. The velocity in the death rate increases, for a given per cent chlorine-demand satisfaction, with an increase in the contact time.

6. Upon storage, greater per cent bacterial reductions result with low per cent chlorine-demand satisfaction.

7. Chloro-peptones and chloro-proteins are neither inhibitory to the growth of bacteria nor germicidal.

8. Addition of as much as 50 p.p.m. of peptone, lactose (NH_4)₂ CO_3 or $\text{NH}_4\text{H CO}_3$ to sewage does not affect to an appreciable extent the per cent bacterial reductions upon chlorinating for 10 minutes to the extent of 50 per cent of the chlorine demand. Additions of 5 cc. of urine influence to a very marked degree the per cent bacterial removal when chlorinated to the same extent.

9. Upon chlorinating sewage, no matter to what extent of

the chlorine demand, chloro-products are formed which bring about bacterial reductions.

10. The chloro-products formed appear to affect the bacterial reductions to a greater extent with increasing contact times.

11. The chlorine demand increases with the increase in the amount of interfering substances.

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AN INVESTIGATION OF THE THERMAL DEATH POINT OF SACCHAROMYCES ELLIPSOIDEUS

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In the preservation of various fruit products such as juices and concentrates it is important to know accurately the effect of length of the heating period and other factors on the death temperature of yeasts, the microorganisms responsible for much of the spoiling of such products. The data published by various investigators on the death temperature of yeasts are not in close agreement. For the above reasons the present investigation was undertaken.

The very early investigators of the eighteenth and nineteenth centuries were principally concerned with proving or disproving the spontaneous generation theory, that is, in discovering whether foods and various infusions did or did not show spontaneous generation after heating at the boiling point in various manners. They did not differentiate to any great extent between the various major groups of microorganisms. Pasteur, however, determined the temperature necessary in the pasteurizing of fermented products such as beer.

Kayser, in 1889, found that yeasts in the presence of moisture were usually killed between 50 and 55°C. and that certain yeasts in the dry condition withstood temperatures of 100 to 120°C. He reported that yeasts spores are killed at a temperature about 5°C. above that necessary for destruction of the vegetative cells.

Nakamura, in 1897, reported that the yeast studied by him was killed at about 52°C. in twenty minutes and at about 50°C. in thirty minutes. He also stated that fermenting power of the yeast was weakened by exposure to 50°C. for a period slightly less than that required for destruction of all the cells.

Cochran and Perkins, in 1914, heated yeasts in syrup and found that heating at 58°C. for thirty minutes did not prevent subsequent fermentation, but that 65°C. for the same period killed all of the yeast cells present.

Wells, in 1918, reported that added sugars and colloids such as starch increase the resistance of yeasts to heat. He found living yeast cells in bread that had reached 88°C. in baking.

Ayers, in 1926, determined that yeasts isolated by him from spoiled catsup survived 60°C. for five minutes, but were killed in ten minutes at this temperature.

Peterson, Levine and Buchanan, in 1927, found that the death time for yeasts isolated from spoiled carbonated beverages was shortened by addition of acid and lengthened by addition of sugar.

Tracy, in 1932, stated that *S. ellipsoideus* cells are destroyed at 62°C. in one minute and at 59°C. in five minutes.

EXPERIMENTAL PROCEDURE

For the majority of the death point determinations concentrated grape juice of 70 per cent total soluble solids content was diluted with distilled water to 15 per cent total soluble solids. In the series of tests on the effect of pH value on the death point, M/15 phosphate buffer media were adjusted to various pH values by varying the proportions of M/15 Na_2HPO_4 , NH_4PO_4 and HCl and were used in comparison with the grape juice medium of corresponding pH values. Grape juice agar consisting of grape concentrate diluted to 10 per cent soluble solids and containing 2 per cent of agar was used in making the plate counts of the cells surviving in the various heating tests.

The yeast used was a strain of *S. ellipsoideus* isolated by one of us (Cruess) in this laboratory, in 1913, from California grapes. It is designated in our collection as *S. ellipsoideus* No. 66. For the death point experiments it was grown at 33°C. on a grape juice agar slant for four to six days for each experiment. Tests proved that the resistance of the cells of this age was at a maximum. One loopful of the agar slant growth was transferred to 9 cc. of the liquid medium to be used in the experiment in question. The

tube containing this stock culture was held in cracked ice and water at 0°C. during each experiment (usually an hour or less) in order that multiplication of the cells would be arrested; this in order that 1 cc. transfers made during the experiment would contain approximately equal numbers of cells.

Tubes containing 9 cc. each of the medium to be used and previously sterilized in test tubes were placed in the heating bath and brought to temperature before adding to each 1 cc. of the stock culture. The heating period was completed. The tubes were then removed and dilution transfers made in tubes of sterile medium of the same kind used in the heating test. This medium was used in preference to the customary sterile water because experiments showed that many yeast cells are killed on dilution of a culture with water. Thus, in a typical test the yeast was exposed in grape juice at 53.9°C. for five minutes and was then diluted in sterile water and in sterile grape juice. Before heating there were present approximately 110,000 living cells per cubic centimeter as shown by plating on agar. After heating, dilution in grape juice showed approximately 25,000 living cells per cubic centimeter and dilution in water approximately 15,000. Other tests gave similar data.

The bath used consisted of a 5-gallon tank of water heated by a gas flame in which the water was stirred by means of compressed air. As previously stated, the tubes of juice or phosphate buffer were placed in the bath and brought to bath temperature before inoculation and transfers for plating were made immediately at the end of each period. All tests were made in duplicate and, naturally, when duplicates failed to agree reasonably well, the tests were repeated in duplicate.

The periods of exposure were ten, twenty, forty, sixty and one hundred and twenty minutes in the lower temperature ranges. At each temperature, qualitative tests were made in addition to the quantitative plate counts; i.e. tubes of the media were chilled immediately after heating and were then incubated to ascertain whether or not growth and fermentation occurred. The temperature interval was in most cases 1°C. between successive tests.

In the experiments upon the effect of carbon dioxide, nitrogen,

and oxygen on the death point of yeast, the liquid to be heated was contained in an 8-ounce tin can fitted with gas-tight connections for admission of gas and for exhausting by suction pump. The container was immersed in the bath and brought to bath temperature before inoculation of its contents. The container was exhausted, the vacuum released by the gas to be used, and the pressure brought to and maintained at the desired point during the heating period.

PRESENTATION AND DISCUSSION OF DATA

In order to conserve space the data are presented in the form of graphs.

Effect of pH value and time of exposure on death temperature. Using the qualitative method, i.e., survival as shown by subsequent fermentation, or complete destruction as shown by subsequent absence of growth, it was found that within certain limits the death temperature of yeast is a function of the time of exposure. This fact is illustrated in figures 1 and 2.

Typical numerical data will emphasize the effect of length of exposure. Thus, in the grape juice of normal pH value (pH 3.8) all the cells present (about 100,000 per cubic centimeter) were killed at 57.5°C. in ten minutes, whereas at 51.5°C. it was necessary to heat the inoculated liquid for eight hours and at 54.7°C. for two hours. When the logarithm of the time was plotted against the corresponding death temperatures a straight line was obtained, indicating that the time required to kill at various temperatures varies exponentially.

While the pH value of the medium affected the death temperature measurably, the effect was much less than that observed with spores of bacteria by a number of other investigators. The yeast cells were more resistant to heat in the neighborhood of pH 4.0 than at pH values much above or below this point. It was not found feasible to make use of the grape juice medium in the alkaline range since the sodium hydroxide reacted with certain constituents of the juice to give substances toxic to yeast. The simple phosphate buffers were used therefore for pH values much above 7.0. However, in most cases in the acid range (pH values

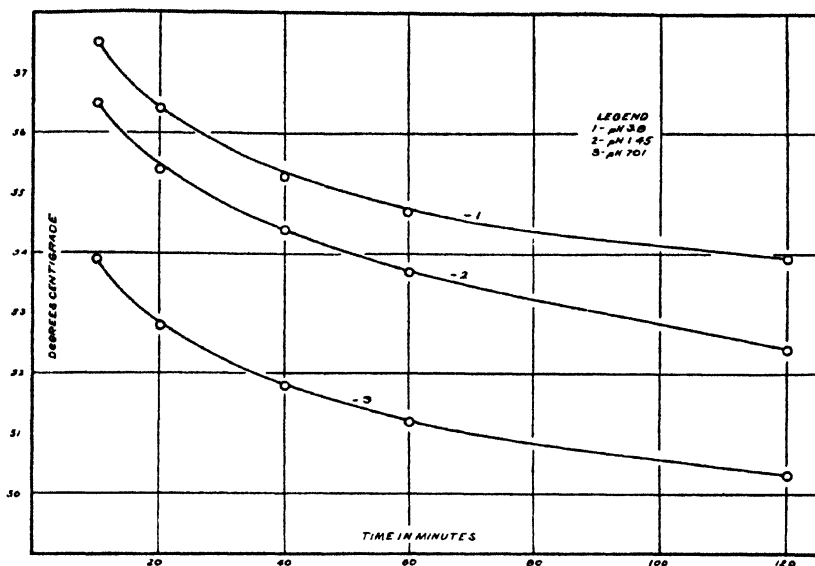


FIG. 1. DEATH TEMPERATURE OF YEAST 66 AS AFFECTED BY LENGTH OF HEATING PERIOD AND pH VALUE OF THE MEDIUM WHEN GRAPE JUICE MEDIUM WAS USED

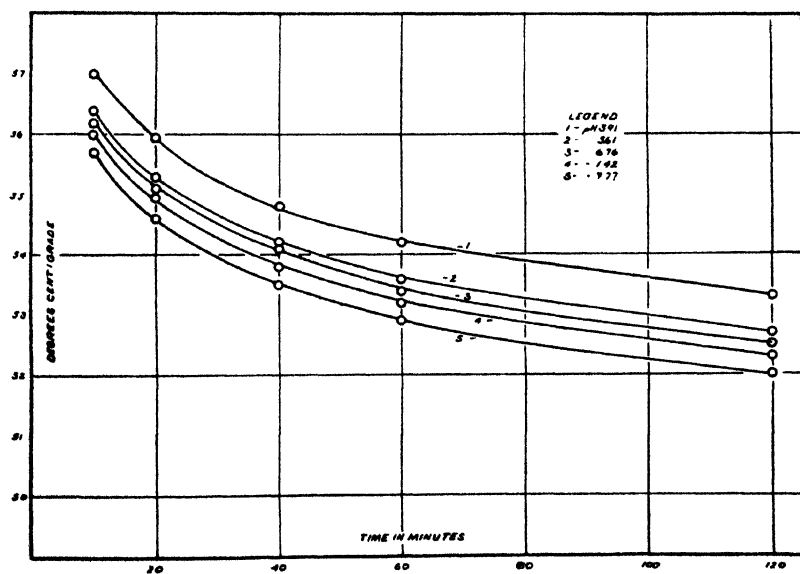


FIG. 2. DEATH TEMPERATURE OF YEAST 66 AS AFFECTED BY THE LENGTH OF THE HEATING PERIOD AND pH VALUE OF THE MEDIUM WHEN PHOSPHATE BUFFER MEDIA WERE USED

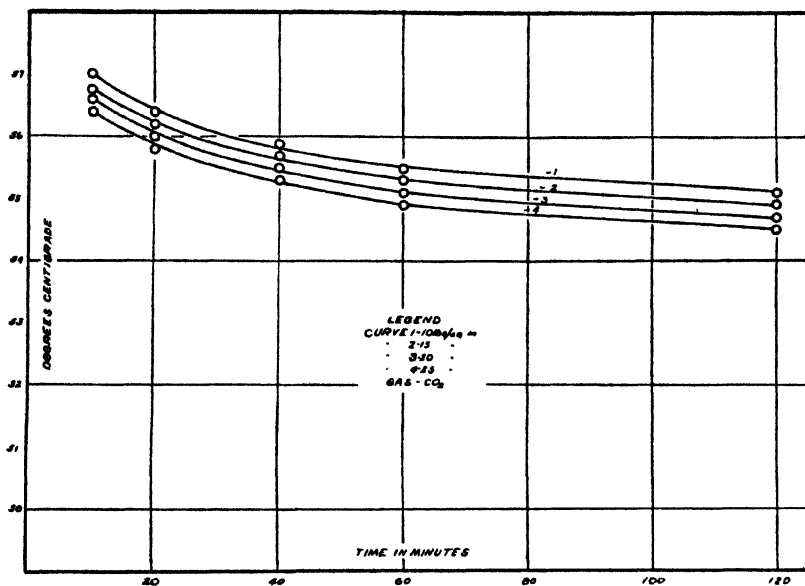


FIG. 3. EFFECT OF CARBON DIOXIDE UNDER PRESSURE ON THE DEATH TEMPERATURE AND DEATH TIME OF YEAST

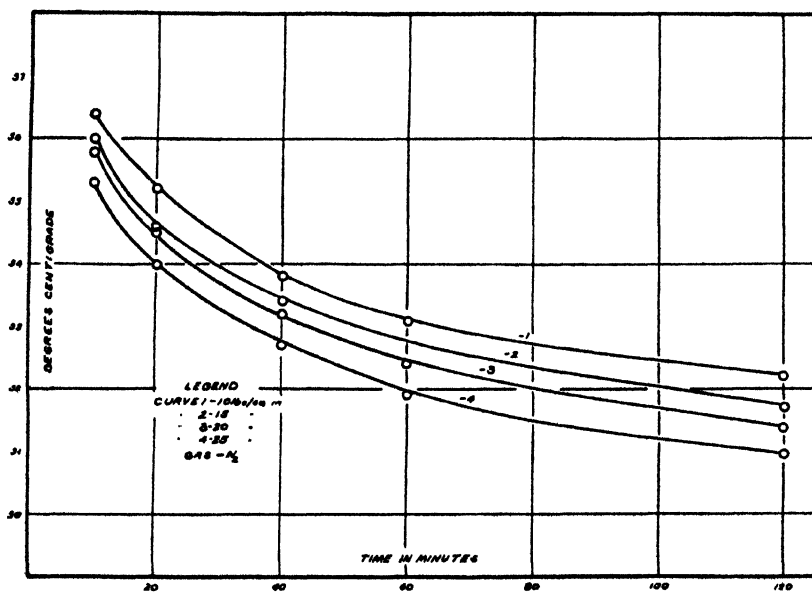


FIG. 4. EFFECT OF NITROGEN GAS UNDER PRESSURE ON THE DEATH TEMPERATURE AND DEATH TIME OF YEAST

below 7) the death temperature of the yeast was slightly lower in the phosphate medium.

The effect of carbon dioxide gas at several pressures on the death temperature is shown in figure 3 and that of nitrogen gas in

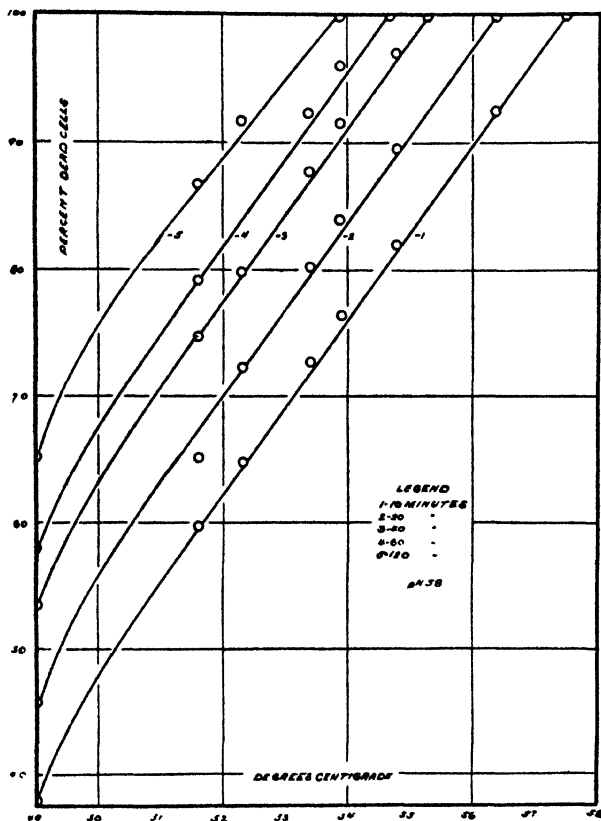


FIG. 5. EFFECT OF TEMPERATURE ON DEATH RATE OF YEAST CELLS IN GRAPE JUICE MEDIUM OF NATURAL pH VALUE (pH 3.8)

figure 4. While these gases under pressure did not greatly lower the death temperature, they did exert a measurable effect in that direction. Nitrogen appeared to be more active than carbon dioxide in this respect.

Thermal death rates. By plating, before and after heating, sus-

pensions of cells in grape juice and in phosphate buffer media counts of living cells were made in order to determine the death rates at various temperatures. Some of the data are presented in figures 5 and 6 in which it will be observed that "per cent

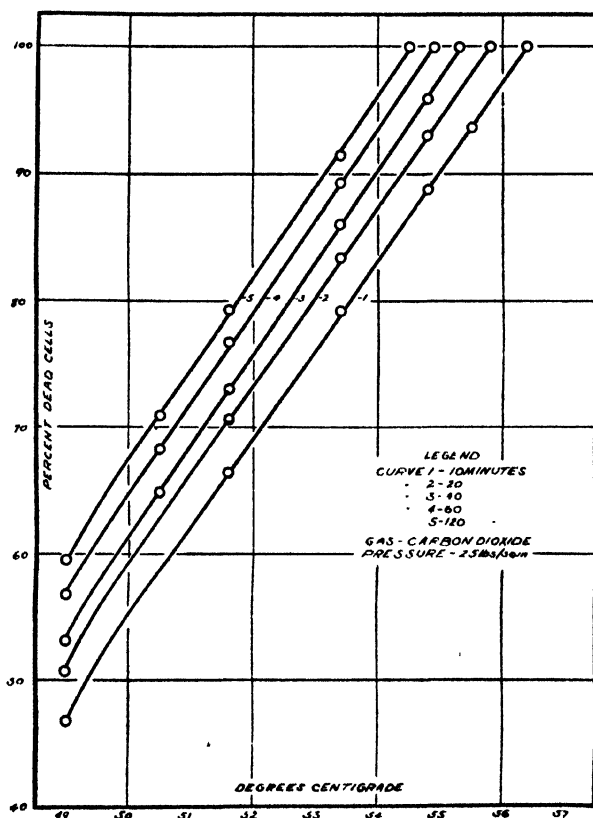


FIG. 6. EFFECT ON DEATH RATE OF YEAST OF CARBON DIOXIDE GAS AT 25 POUNDS PRESSURE

killed" (per cent dead cells) has been plotted against the temperature, a separate curve being given for each heating period; i.e., a curve for ten minutes, one for twenty minutes, etc. As an example, it will be seen that at 54°C. about 75 per cent of the cells were killed in ten minutes, about 84 per cent in twenty minutes,

about 92 per cent in forty minutes, about 96 per cent in sixty minutes, and 100 per cent in one hundred twenty minutes. At 49°C. the observed values in this series of tests were about 38 per cent killed in ten minutes, 53 per cent in forty minutes, about 58 per cent in sixty minutes, and about 65 per cent in one hundred twenty minutes. In other words, the slope of the curve is less at the lower temperature.

The effect of the pH value on the rate of kill may be illustrated by the following data. At pH 3.8 and 49°C., with twenty minutes heating, 45.8 per cent of the cells were killed; at pH 1.45 and twenty minutes heating, 53.15 per cent were killed. For other times and temperatures, also, the per cent of cells killed was somewhat greater at pH 1.45 than at 3.8. At pH 7.0 and twenty minutes heating at 49°C., 81.4 per cent of the cells were killed in grape juice. However, in the phosphate buffer medium the difference in the rates of destruction at pH 3.91 and 6.76 were less than was the case between pH 3.8 and 7.0 in grape juice. Possibly neutralization of the grape juice with NaOH to pH 7.0 formed some slightly toxic substance which increased the death rate of the yeast. As a matter of fact, in order that the juice would have a pH value of 7.0 after sterilization of the medium in tubes at 100°C. it was necessary to add a considerable excess of NaOH and this undoubtedly caused appreciable decomposition.

SUMMARY

1. It was found that the death temperature of a strain of wine yeast, *Saccharomyces ellipsoideus* from California grapes varies to a considerable degree with the length of the heating period, being in grape juice of pH 3.8 approximately 57.5° for ten minutes heating and approximately 54°C. for one hundred twenty minutes heating.

2. The pH value of the medium affects the death temperature and the death rate relatively little over the range of pH 1.45 to 7.0. The yeast is slightly more resistant to heat at pH 3.8 to 4.0 than at pH values much above or below 4.0.

3. Nitrogen gas and carbon dioxide gas under 5 to 25 pounds

pressure lower the death temperature and increase the death rate in measurable, but not in marked, degree.

4. From the data obtained in these experiments and from practical scale pasteurization tests not reported in this paper with bottled and canned juices, we would state that it is feasible to pasteurize fruit juices successfully at 54.5 to 55°C. (approximately 130 to 131°F.).

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THE BACTERICIDAL PROPERTIES OF ULTRAVIOLET IRRADIATED PETROLATUM

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In a preliminary paper from this laboratory (Ross, 1932) it was shown that an ultraviolet irradiated petrolatum-lanolin mixture had marked bactericidal power for *Staphylococcus aureus* while the same mixture without irradiation possessed no such quality. *B. pyocyaneus* exhibited considerable resistance to the destructive effect of the irradiated mixture. Several investigators have reported the acquisition by the fatty oils of bactericidal properties upon being irradiated. In this paper we desire to report our investigation of the comparative effect upon a number of bacterial species of ultraviolet irradiated and non-irradiated petrolatum without the admixture of the fatty oils. Some previous reports on this subject appear in the literature. Eising (1931) in discussing the therapeutic use of irradiated petrolatum, states that its effect is "dependent upon a strong bactericidal action upon the ordinary organisms of wound infection together with a potent stimulus to the processes of healing." He carried out no direct *in vitro* experiments, however, to demonstrate bactericidal action but assumed such from the observation that when petrolatum was applied directly to the infected wound "the discharge changed from a purulent to a serous one and the bacterial count diminished rapidly." Thompson and Sheard (1931) reported that a number of oils, including petrolatum and lighter mineral oils, became bactericidal after irradiation from an ultraviolet source.

THE BACTERICIDAL ACTIVITY OF IRRADIATED PETROLATUM

In our tests, old laboratory strains of the bacteria were mixed directly with the material the bactericidal power of which it was

desired to determine. The physical properties and presumably the chemical composition of samples of petrolatum obtained at random from drug houses vary greatly. In this problem we were interested only in the nature and diversity of the killing power of the irradiated oil and made no attempt therefore to discover the raw product which would yield the most bactericidal end substance. Preliminary tests indicated that a preparation having a melting point of from 40 to 45°C. gave somewhat more satisfactory results than samples melting at higher temperatures. The lower melting material also presented fewer difficulties in handling. We therefore procured a large quantity at one time and used the same sample in all our tests.

Heat-sterilized petrolatum was delivered to ordinary 100 mm. Petri dishes in 15 cc. amounts. The dishes were then placed under a Burdick quartz tube lamp at a distance of 12 inches with covers removed. The lamp developed a temperature of about 56°C. in the petrolatum which therefore remained melted during irradiation. The white petrolatum changed in color under the influence of the ultraviolet rays. In four hours it became lemon yellow. Longer periods of exposure produced a deep butter-yellow color. Experiments with products irradiated under the same conditions for varying lengths of time seemed to indicate that the maximum bactericidal power was obtained with our set-up in about four hours. All of the detailed results discussed in this paper were carried out with material irradiated for that length of time.

Obtaining an even distribution of the organisms in the petrolatum offered some difficulties but it was found that a quite uniform mixture could be obtained in the following way: Fifteen-cubic-centimeter portions of petrolatum in Petri dishes were warmed until melted and subsequently maintained at a temperature of 45°C. One-half cubic centimeter amounts of twenty-four hour broth cultures of the organisms were added from a pipette and stirred into the petrolatum with a platinum wire. Stirring continued until the petrolatum had cooled and solidified. The plates were then covered and placed in the incubator at 37°C. At intervals rather large portions of the material were removed

with a loop and melted off into tubes of broth which had been previously warmed to approximately 45°C. The broth tubes were incubated for forty-eight hours and the presence or absence of growth noted. The kind of medium used for the growth of

TABLE 1

Comparative bactericidal tests with four-hour ultraviolet irradiated and non-irradiated petrolatum

ORGANISM	GROWTH AFTER CONTACT FOR VARYING PERIODS WITH IRRADIATED PETROLATUM (HOURS)										GROWTH AFTER CONTACT FOR VARYING PERIODS WITH NON-IRRADIATED PETROLATUM (HOURS)									
	2	3	4	5	12	13	24	48	72	168	2	3	4	5	12	13	24	48	72	168
<i>Staph. aureus</i> No. 1	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-
<i>Staph. aureus</i> No. 0	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-
<i>Strep. viridans</i>	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-
<i>Strep. non-hemolyticus</i> (Clawson's strain)	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-
<i>Strep. hemolyticus</i>	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-
<i>C. diphtheriae</i>	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-
<i>C. Hofmanni</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Bact. coli</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Bact. typhosum</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Bact. paratyposum</i> A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Bact. paratyphosum</i> B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Bact. dysenteriae</i> , Flexner	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Bact. mucosus-capsulatus</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>B. pyocyaneus</i> No. 199a	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+
<i>B. pyocyaneus</i> No. 195	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
<i>Br. abortus</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Myc. tuberculosis</i> (Novy)	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
<i>Myc. smegmatis</i>	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
<i>N. catarrhalis</i>	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
<i>Cl. Welchii</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Cl. tetani</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>B. anthracis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>B. subtilis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

the organisms both before and after mixing them with the petrolatum varied with the organism used. Meat-extract broth was used for those organisms which multiply readily in that medium. Meat infusion was used for the streptococci and diphtheroids. For the tubercle bacillus Lubenau's coagulated egg medium was

employed to produce the culture, which was then suspended in broth before being mixed with the petrolatum. After exposure it was again inoculated to Lubenau's medium to test for viability. Incubation of these latter cultures was continued for two weeks before any test was called negative.

In table 1 are given the results of these tests. It will be seen that the only organisms we failed to recover from the non-irradiated petrolatum after quite long periods of exposure were the diphtheroid, *C. Hofmanni*, and *Brucella abortus*. It would appear that these organisms are killed by the raw product. Of the organisms which survived long contact with the non-irradiated oil but were killed by the four hour irradiated product, the diphtheria bacillus and a strain of *Streptococcus hemolyticus* succumbed in the shortest time. Live organisms were proven in these cases after two hours but not after three hours. The spore-forming aerobic and anaerobic organisms proved to be insusceptible to the product, surviving the maximum period tested, seventy-two hours. Perhaps the most striking feature observed in these results is the insusceptibility of the Gram-negative intestinal group of bacteria. All of these strains survived the maximum period of exposure tested, which was in no case less than twenty-four hours and in the cases of *Bact. coli* and *Bact. typhosus* as high as one hundred sixty-eight hours. Of the two strains of *B. pyocyaneus* one was killed in twenty-four hours and the other survived that period but was not viable at the end of forty-eight hours. The tubercle bacillus tested was the non-pathogenic, rapidly-growing Novy strain. It was not recovered after forty-eight hours admixture with the irradiated petrolatum though positive cultures were obtained at the end of twenty-four hours. The strain of the smegma bacillus used could not be cultured after twenty-four hours' exposure.

We were unable to devise any accurate quantitative method of testing the killing power of the petrolatum. A rather crude method was attempted with the colon bacillus in order to determine whether this organism was wholly insusceptible to the bactericidal power of the irradiated petrolatum or whether failure to procure sterilization was due to the extraordinary resist-

ance of some individuals only in the cultures. This test consisted in mixing the organisms with the petrolatum in the manner already described, then at intervals removing a portion of the material roughly measured with a very small aluminum spoon and melting it off into 10 cc. of warm broth. After thorough mixing measured portions of this broth were plated in duplicate. The colonies developing on the plates were counted after forty-eight hours' incubation. A number of these tests were carried out and while the figures obtained show clearly that the method was only very roughly quantitative, the differences obtained between the irradiated and non-irradiated samples were in general no greater than those between two tests on the same sample at different times and indicated, we believe, that *Bact. coli* remains unaffected by the irradiated petrolatum for the duration of the test, twenty-four hours.

EXPERIMENTS ON THE PROPERTIES OF THE GERMICIDAL AGENT IN IRRADIATED PETROLATUM

There seem to have been three hypotheses advanced to account for the bactericidal effect of ultraviolet irradiated oils. Wrenn (1927) obtained killing of *Staphylococcus aureus* inoculated upon the surface of agar plates when the latter were exposed at some distance to dishes containing various irradiated fatty oils. He believed the killing effect to be due to emanations of radiant energy stored in the oils during irradiation. Eising (1931) in his first paper on the bactericidal properties of irradiated petrolatum expressed his belief also that these properties were resident in secondary rays emanating from the irradiated material. In his later papers (1932, 1933), however, he repudiates this belief in favor of the supposition that a chemical non-gaseous substance, possibly of the nature of the sterols, is the responsible agent. Harris, Bunker and Milas (1932) carried out experiments similar to Wrenn's, chiefly with the vegetable oils, and concluded that the killing effect exerted at a distance by the irradiated products was due to volatile chemical compounds and not to secondary emanations of radiant energy. With mineral oils they obtained no killing by their method.

The reasoning leading to the radiant energy hypothesis has been considerably affected by the fact that both vegetable and mineral oils when irradiated acquire, along with their bactericidal powers, the property of affecting photographic plates exposed to them in the dark room. Eising (1931) and French (1932) have published clear-cut stencil designs obtained both by direct exposure of plates to irradiated petrolatum and by exposure through an x-ray film to which the photographic plates were hermetically sealed. Harris, Bunker and Milas (1932) believe that they have shown this effect, at least so far as the vegetable oils is concerned, to be due to the presence of peroxidic compounds given off from the irradiated products in vapor form. They believe these peroxidic vapors are likewise responsible for the bactericidal effects of the oils. It appears, however, from a personal communication from these authors cited by Eising (1932) that they have been unable to detect any peroxidic oxygen given off by irradiated petrolatum though they have obtained fogging of photographic plates even when the latter were protected by x-ray film screens. To accept a gaseous emanation as the direct cause of the fogging of the plates would necessitate the assumption that such a gas could pass through the x-ray film. This has been suggested by Eising (1932) as probably occurring. The latter author (1933) seems to incline to the opinion, however, that the effect upon photographic plates and the bactericidal action of irradiated petrolatum are due to different constituents of these preparations.

The results of our experiments upon the problem of the nature of the germicidal agent in ultraviolet irradiated petrolatum are by no means conclusive, but we believe they throw some light upon the subject.

We were unable, as were Thompson and Sheard (1931), and Harris, Bunker and Milas (1932), to obtain killing by exposure of the organisms to irradiated petrolatum without actual contact between the organisms and the oil. Using the method of Harris, Bunker and Milas, surface streaks on agar plates were made of *Staphylococcus aureus* and placed uncovered over dishes of the irradiated petrolatum. Growth invariably occurred on these

plates. Later, in order to rule out possible protective effects by the medium, drops of a water suspension of the organism were placed on sterile glass slides which were then inverted over the petrolatum at distances no greater than 1 or 2 mm. The dishes containing these tests were placed in a moist chamber to prevent drying. Some were held at room temperature, some at body temperature, for periods up to forty-eight hours. None showed dependable evidence of being affected by the petrolatum. When subcultured after exposure they always showed abundant growth. Drops of a water suspension dried upon slides and exposed in the same way likewise remained viable for twenty-four- and forty-eight-hour periods.

A somewhat different experiment intended to test the killing power of the irradiated petrolatum when not in contact with the exposed organisms was carried out as follows:

A Petri dish was partly filled with the irradiated petrolatum and warmed until just melted. By means of a syringe or a capillary pipette droplets of a water suspension of *Staphylococcus aureus* were injected into it. The plate was then quickly cooled. By exercising care it was possible to obtain droplets of the suspension imprisoned in the solidified oil ranging from 3 or 4 mm. in diameter to a size that was barely visible. By covering the surface with another layer of the petrolatum these droplets would remain stationery indefinitely. The plates were placed in the incubator at 37°C. and at the end of twenty-four hours the individual droplets were cut out with a sterile wire and placed in broth warm enough to melt the petrolatum. The tubes were incubated for forty-eight hours and growth noted. Accurate measurements of the droplets were not attempted but a number of tests indicated that in the irradiated petrolatum the smaller ones were invariably sterilized while the larger ones were not. We would estimate the maximum size of the droplets which were rendered sterile to be not in excess of 1 mm. in diameter. In the non-irradiated material none of the droplets were sterilized.

The results of this last experiment were at first interpreted as favoring the hypothesis that the bactericidal power lay in the action of secondary radiant energy emanating from the irradiated

petrolatum, the reasoning being that the amounts of this hypothetical energy available for each organism in the smaller droplets would be many times greater than that impinging upon them when exposed from a plane surface. The position of many of the organisms in the larger droplets would, of course, approach that of those exposed to the radiant plane and hence it did not seem inconsistent with this hypothesis that the larger droplets were not sterilized while the smaller ones were.

Direct experiment, however, to detect possible radiant energy emanations yielded negative results. We obtained, as have others, definite fogging of photographic plates when the latter were exposed directly to the irradiated petrolatum, but when the films were screened behind ordinary glass, quartz glass or cellophane, no fogging could be detected. The last two substances permit the passage from the ultraviolet lamp of the rays which confer the bactericidal powers upon the petrolatum as was shown by the fact that petrolatum irradiated through them assumed the characteristic yellow color and became bactericidal for *Staphylococcus aureus*.

From these experiments it seems probable that the effect upon photographic plates exposed to irradiated petrolatum is due to gaseous emanations from this material. So far, however, there seems to be no evidence that such emanations are in any degree germicidal. It would appear then that the germicidal agent must be sought in some non-volatile chemical substance formed in the petrolatum under the influence of the rays from the ultraviolet lamp.

That a chemical reaction takes place in the petrolatum during irradiation would be assumed from the change in color. This assumption is supported by the results of the following experiment which further suggests that the change involves oxidation. That such oxidative change is correlated with both the appearance of the yellow color and the attainment of bactericidal power appears also from this experiment.

Two quartz glass tubes were sterilized and petrolatum from the same sample used throughout these tests was placed in each. One tube was filled completely full and corked tightly with a cork

stopper which was sealed in with sealing wax. No air remained in the tube. The other tube was only half filled and was stoppered with a cork having a portion removed to permit free access of air. These two tubes were placed on their sides under the ultraviolet lamp and irradiated for four hours. The tube from which air was excluded underwent no change in color under the influence of the rays but the other assumed the same lemon yellow color always acquired by the petrolatum irradiated in the open Petri dishes. Upon subsequent exposure to air the first tube retained its original white appearance. Bactericidal tests on the two samples showed that the material irradiated in the absence of air had acquired no bactericidal powers for *Staph. aureus* while the sample irradiated through the quartz glass in the presence of air sterilized a culture of *Staph. aureus* in six hours when mixed with it in the manner described above. In a second test the same results were obtained except that seven hours were required for the petrolatum irradiated in the open tube to sterilize the culture.

Long ago Roux (1887) showed that anthrax spores would not germinate in a medium that had been exposed for several hours to direct sunlight. Later investigations by Burnet (1925) indicated that this was due to the presence in the medium of hydrogen peroxide formed by the oxidation of water under the influence of the ultraviolet rays. We shook up melted irradiated petrolatum repeatedly with warm water and tested the water for the presence of hydrogen peroxide. None was found. The addition of the water to equal quantities of broth also failed to render the latter inhibitive to the growth of staphylococci. Extraction of the petrolatum with warm broth in a similar manner added nothing to the broth that would inhibit the growth of staphylococci. Apparently the bactericidal agent in this material is not extractable with water or with broth. This would seem to rule out compounds such as peroxides, acids, bases and even ozone. The latter compound would likewise be ruled out by the fact that the irradiated petrolatum retained its bactericidal power for *Staphylococcus aureus* after having been heated to temperatures of 50 to 60°C. for several hours.

We also attempted to wash out any volatile bactericidal sub-

stance from the irradiated petrolatum by allowing air to bubble slowly through a flask of the substance (which was melted and kept at a temperature of 60°C.), and subsequently to bubble through a flask of broth. After twelve hours of this washing the broth remained without any growth-inhibiting effect on *Staphylococcus aureus* and the irradiated petrolatum appeared to have lost none of its bactericidal power. In a somewhat similar experiment water was distilled from a mixture of irradiated petrolatum and water, the distilling flask being constantly agitated to facilitate mixing. The distillate was tested for bactericidal effect on *Staphylococcus aureus* and was found negative.

We next attempted to remove the bactericidal substance by extraction with alcohol, ether and petroleum ether. These tests were on the whole unsatisfactory but the results with alcohol are worth recording. When melted irradiated petrolatum is shaken up with warm alcohol and then allowed to stand, two layers form. The lower layer is yellow and appears little different from the irradiated petrolatum alone. The upper is only slightly tinged with yellow and when evaporated on a water bath, leaves a very small residue. This is brownish in color and on cooling has the appearance of a heavy oil rather than a semi-solid material like petrolatum. Both the extracted material (the lower layer) and the brownish residue, when freed from alcohol as completely as possible by evaporation, were bactericidal for *Staphylococcus aureus*.¹ Since small amounts of alcohol may have remained in both specimens, however, we hesitate to infer that the bactericidal substance is alcohol-soluble.

SUMMARY AND CONCLUSIONS

The experiments reported in this paper show that when white petrolatum is irradiated for four hours from a source of ultra-violet light it changes to a lemon yellow color and acquires the property of slowly sterilizing suspensions of certain bacteria, when intimately mixed with them. The bactericidal effect thus

¹ By a special method several ounces of the alcohol extractive were obtained and used clinically by Dr. Arthur Jones who believed it to be therapeutically more potent than the ordinary irradiated product.

demonstrated appears to be selective in the sense that it acts upon certain vegetative forms of bacteria only. Spores are not destroyed and certain Gram-negative intestinal organisms seem to be likewise wholly unaffected.

Evidence is brought out to indicate that the germicidal agent in this ultraviolet irradiated petrolatum is a non-volatile chemical substance rather than a gaseous or radiant energy emanation and that this substance is formed as the result of an oxidative process. It is further shown that this chemical agent is not extractable with water or with ordinary nutrient broth and that it cannot be carried out of the melted material with a stream of air or distilled out with steam. There is some indication that the active bactericidal agent is extractable with alcohol, but we do not feel that our experiments are conclusive on this point.

It seems to us a reasonable conclusion from the above facts that irradiated petrolatum contains a very weakly germicidal substance of such low solubility and slow diffusibility that it can be depended upon to kill susceptible bacteria only when the organisms and the petrolatum are brought into very intimate contact. To expect this substance therefore to exert any effective bactericidal action upon organisms contained in pus or exudates or embedded in tissue, conditions which would certainly prevent the necessary contact, is illogical. It is much more logical to suppose that any action which irradiated petrolatum may show in freeing infected wounds of their organisms is due to a stimulating effect upon the local tissue defenses rather than to direct bactericidal action.

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THE FERMENTATION OF SORBITOL AND TREHALOSE BY HAEMOLYTIC STREPTOCOCCI FROM VARIOUS SOURCES

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Many attempts have been made to differentiate haemolytic streptococci of human and animal origin. Experiments by Ayers, Johnson and Davis (1918), Avery and Cullen (1919), and Brown (1920), show that the final hydrogen ion concentration of a 1 per cent glucose broth culture is usually higher for streptococci of bovine origin than for those of human source. In this connection, it is important to use slightly alkaline plain nutrient broth plus glucose, since Jones (1920) has shown that streptococci produce more acid in the presence of ascitic fluid and Foster (1921) found less acid produced in broth having an initial acidity of pH 6.2 than in broth with an alkalinity of pH 7 to 8. Ayers and Rupp (1922) and Reis and Swensson (1931) were able to separate streptococci of bovine source from those of human origin by their ability to hydrolyze sodium hippurate. Sherman and Albus (1918) showed that all strains which reduced methylene blue were of bovine origin. Avery (1929) showed that exposure to methylene blue killed practically all human and bovine strains which did not reduce the dye. Edwards (1932) found that certain strains of animal origin could not be distinguished from those of human origin by the usual methods, but that a differentiation could be made by the fermentation reactions of sorbitol and trehalose.

We attempted to confirm Edwards' findings in a much larger group of haemolytic streptococci from human sources and in a smaller group of animal origin. Fermentation reactions were performed with eight carbohydrates, using 328 strains of human and

18 strains of animal origin. In addition, these strains were tested for their ability to hydrolyze sodium hippurate, and for acid production in glucose broth. The source of the cultures is given in table 1.

TABLE 1
Source of cultures

	NUMBER	TOTAL
Strains from human sources		
Scarlet fever cases { Blood.....	1	
{ Throats.....	13	
{ Secondary lesions.....	5	
{ Surgical.....	3	
Contacts—throats.....	9	
Carriers—throats.....	3	
Stock cultures (N. Y. 5, Dick I, II, III, IV).....	5	39
Erysipelas.....	11	11
Puerperal sepsis cases { Blood.....	10	
{ Cervix.....	27	
{ Abscess.....	2	
Contacts—throats.....	6	45
Kirkland Lake septic sore throat epidemic (1930):		
Cases and convalescents—throats.....	20	
Diagnosis uncertain—throat.....	1	
Contacts—throat.....	1	
School group—throats.....	22	
Milk handlers—throats.....	9	
Lee septic sore throat epidemic (1928):		
Case—Lee 76—blood.....	1	54
Miscellaneous { Streptococcal infections.....		
{ Normal throats.....	9	53
Institution O { Dick positive children—throats.....		
{ Dick negative children—throats.....	16	29
Institution B—throats.....	97	97
Total strains from human sources.....		328

TABLE 1—*Concluded*

		NUMBER	TOTAL
Strains from animal sources			
Bovine	{ Lee septic sore throat epidemic (1928) Lee 29A—milk.....	1	
	{ Kirkland Lake septic sore throat epidemic (1930—milk).....	4	
	{ Boston septic sore throat epidemic (1917)—milk.....	1	6
Guinea pigs	{ Epizoötic lymphadenitis.....	3	
	{ Blood.....	1	4
Horse—strangles-abscess		2	2
Rabbit	{ Blood.....	5	
	{ Brain.....	1	6
Total strains from animal sources			18

HYDROLYSIS OF SODIUM HIPPURATE

Sodium hippurate medium made according to the method of Ayers and Rupp (1922) or of Hardenbergh (1930) was dispensed in 5 cc. quantities and seeded with a loopful of 24-hour serum-broth culture of the strain to be tested. The addition of 0.5 cc. of a 7 per cent aqueous solution of ferric chloride to 2 cc. of 72-hour culture produced a precipitate if sodium hippurate had been hydrolyzed. The most satisfactory readings for the evidence of hydrolysis were made after the tubes containing culture and ferric chloride had stood overnight to permit the re-dissolving of any precipitate other than the benzoate. Then the supernatant liquid was either clear or uniformly turbid, the latter condition showing that hydrolysis had occurred.

Among the animal strains, 3 hydrolyzed sodium hippurate, 1 from the brain of a rabbit which had been inoculated intracerebrally with vaccine virus from a calf, and 2 from the milk of cows examined during the Kirkland Lake epidemic of septic sore

throat. Cultures from the milk of 2 other cows concerned in the Kirkland Lake epidemic, strain C 108 from the Boston epidemic of 1917, and strain Lee 29 A from the Lee epidemic of 1928 failed to hydrolyze sodium hippurate. Our thanks are due to Dr. J. H. Brown for strain C 108 and to Dr. Elliott Robinson for strain Lee 29 A. Only 49 of the human strains hydrolyzed sodium hippurate, 2 strains from the throats of milkers in the Kirkland Lake epidemic, and 47 strains from the throats of deaf children living in Institution B. In this institution cultures of haemolytic streptococci were found in 121 of the 143 throat swabs which were taken about a week after the occurrence of several cases of scarlet fever. It is quite possible, since as far as we were able to determine, the milk supply obtained from their own herd was not pasteurised, that some of these strains were actually of bovine origin.

ACID PRODUCTION

Meat infusion broth containing 1 per cent peptone, 0.5 per cent sodium chloride and 1 per cent glucose adjusted to pH 7.8 was dispensed in 5 cc. quantities and seeded with a loopful of 24-hour serum-broth culture of the strain to be tested. Acid production was determined after 48 hours' incubation at 37°C. by comparison with standard buffer solutions. Methyl red was used as indicator and results were usually checked with brom-cresol-green. All human strains except the 49 which hydrolyzed sodium hippurate and two additional raffinose-fermenters from normal throats, produced a final hydrogen ion concentration of pH 5.0 to 5.2. The exceptions just noted as well as the 3 animal strains which hydrolyzed sodium hippurate, produced a final hydrogen-ion concentration of pH 4.4 to 4.6. The remaining 15 animal strains behaved as did the majority of the human strains as regards acid production (see table 2).

FERMENTATION REACTIONS

The medium consisted of meat infusion broth made sugar-free by fermentation with *B. coli*, 1 per cent peptone, 0.5 per cent sodium chloride, 1 per cent Andrade's indicator and 1 per cent of

one of the following carbohydrates: lactose, sucrose, salicin, mannitol, raffinose, inulin, sorbitol, and trehalose. Each medium, seeded with a loopful of 24-hour serum-broth culture of the strain under test, was incubated at 37°C. for seven days, unless fermentation occurred earlier. The purity of the cultures after growth in carbohydrate media was verified by streaking on

TABLE 2

Action of haemolytic streptococci on sodium hippurate and on glucose meat infusion broth

STRAINS FROM	UTILIZATION OF SODIUM HIPPURATE		FINAL HYDROGEN ION CONCENTRATION	
	Hydrolyzed	Not hydrolyzed	pH 4.4 to 4.6	pH 5.0 to 5.2
Human sources.	49	279	51	277
Animal sources.	3	15	3	15

Of 279 strains from human sources which failed to hydrolyze sodium hippurate, only two produced a hydrogen ion concentration of pH 4.4 to 4.6.

TABLE 3

Fermentation reactions of haemolytic streptococci

STRAINS FROM	LACTOSE		MANNITOL		RAFFINOSE		SALICIN		TREHALOSE		SORBITOL	
	+	-	+	-	+	-	+	-	+	-	+	-
Human sources.	321	7	9	319	2	326	284	44	328	0	0	328
Animal sources.	16	2	0	18	0	18	18	0	9	9	9	9

The only strains fermenting sorbitol were of animal origin. They produced in glucose broth a final hydrogen ion concentration of pH 5.0 to 5.2, and did not hydrolyze sodium hippurate.

blood agar plates and by microscopic examination of films of culture stained with Gram's stain. Inulin was not fermented by any strain and sucrose was fermented by all the strains. Table 3 shows the fermentation reactions obtained.

All strains of human and of animal origin fermented trehalose and not sorbitol, except 9 strains of animal origin which fermented sorbitol and not trehalose. These 9 strains fell into the pyogenes group (Holman's (1916) classification), and resembled human

strains in non-hydrolysis of sodium hippurate and low acid-production. The sorbitol-fermenting group comprised all the strains from guinea pigs, four from rabbit's blood, and one from a horse. Referring to the 9 strains of animal origin which were not differentiated from human strains by the fermentation of trehalose and not of sorbitol, 3 were typically bovine by hydrolysis of sodium hippurate and by acid production, and the other 6 could not be differentiated from human strains by any of the tests applied. Four of the 6 animal strains which were indistinguishable from human strains were from milk of cows incriminated in epidemics of septic sore throat, 1 was from rabbit's blood and 1 from a horse. Of the 2 animal strains belonging to the equi group, one hydrolyzed sodium hippurate and produced a high concentration of acid, and the other was typically human by all tests. Among the human strains, 268 belonged to the pyogenes group, 7 to the equi group, 9 to the infrequens group, and 44 to the anginosus group. Forty-two of the anginosus group and 7 of the pyogenes group hydrolyzed sodium hippurate and produced a high concentration of acid. Other than these 49 strains, together with 2 additional strains producing a high acid concentration, all of the human strains would be classified as such by any one of the differentiating tests applied.

DISCUSSION

Of the 328 strains of human origin, 279 or 85 per cent could be classified as human by their failure to hydrolyze sodium hippurate, 277 or 84 per cent could be similarly classified by their relatively slight acid production in glucose broth. Forty-nine strains could not be classified as human by either of these tests. However, the strains of human origin without exception fermented trehalose and not sorbitol. This result is in agreement with the work of Edwards. The 49 strains of human origin which would be classified as bovine by the usual tests were from two milkers and from inmates of one institution where, as far as we were able to ascertain, raw milk was used. A precipitin test on 30 of these cultures, carried out according to Lancefield (1933) confirmed the classification obtained by the other tests; namely, that they re-

sembled strains of animal origin. Hence, the findings support the hypothesis that these strains, although obtained from human sources, had been transferred through contact with cows or by the use of raw milk and were actually of bovine origin.

Of the 18 strains of animal origin, 3 could be differentiated from human strains by the production of a high hydrogen-ion concentration in glucose broth and the hydrolysis of sodium hippurate, 9 others by the fermentation of sorbitol, and 6 resembled human strains in every respect.

In a series of 90 animal strains, Edwards found 5, or 6 per cent which resembled human strains in all their characteristics. In our series the high proportion of animal strains resembling human strains may be attributed to the small number of cultures. It is of particular interest that 4 of the 6 strains resembling those of human origin were from cows incriminated in epidemics of septic sore throat. This observation is in accordance with the accepted view that haemolytic streptococci causing septic sore throat are essentially of human rather than of bovine origin.

SUMMARY

Of 328 strains of human origin, all fermented trehalose but not sorbitol; in both acid-production and hydrolysis of sodium hippurate, 49 of these resembled strains of bovine origin.

Of 18 strains of animal origin, 9 could be identified as such by the fermentation of sorbitol, 3 additional strains by both acid-production and hydrolysis of sodium hippurate; 6 strains could not be differentiated from human strains by the methods used; 4 of the latter were from the milk of cows incriminated in epidemics of septic sore throat.

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THE ACTION OF CERTAIN BACTERIA ON SOME SIMPLE TRI-GLYCERIDES AND NATURAL FATS, AS SHOWN BY NILE-BLUE SULPHATE¹

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In studies on the metatrophic bacteria, action on carbohydrates and proteins has been given much more attention than action on fats. This has been due, in part, to the inability of many of the common bacteria to attack fats, but the relative inconvenience of determining lipolytic action has been a factor also. It appears that more attention to the action of bacteria on fats should yield information of importance in studies on the deterioration of various foods and also in investigations on the systematic relationships of the organisms.

HISTORICAL

In 1894, Sommaruga studied the lipolytic ability of micro-organisms by growing them on solid media in which was dispersed 2.0 per cent olive oil or other fat. No indicator was used, and the disappearance of the fat globules from the region surrounding the growth of the bacteria was accepted as evidence of hydrolysis.

Eijkman (1901) inoculated melted agar and poured it over a thin layer of tallow in a petri dish. Lipolytic organisms formed colonies that caused clear zones in the tallow beneath them.

The simultaneous staining of neutral fats and fatty acids by oxazine dyes was studied by Smith (1908). This investigator pointed out that fat globules contained in the tissues could be stained by basic aniline dyes if they were first changed into fatty

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acids. When an aqueous solution of Nile-blue sulphate was shaken with olein the fat became red, but when shaken with oleic acid the fatty acid became blue or bluish purple, depending on the relative amounts of blue and red substances present in the solution of the dye. The blue color was due to the blue soap resulting from the reaction between the fatty acid and the oxazine base.

Sayer, Rahn and Farrand (1908) detected lipolysis by bacteria with a litmus agar, prepared from sugar-free broth, in which was dispersed a small amount of butter fat. Lipolysis was indicated by a change in the color of the indicator.

Boeminghaus (1920) studied the color reactions of Nile-blue sulphate with palmitin, stearin and olein, their corresponding acids, and certain other derivatives; some natural fats were also used. He emphasized the intense colors secured with oleic acid and its ester combinations (glycerol and cholesterin), the free acid being blue and the combinations red. Palmitic and stearic acids and palmitin and stearin were only slightly colored. In connection with the intense color of oleic acid, compared to other fatty acids, this investigator noted that oleic acid is an unsaturated acid.

Buchanan (1921) pointed out that lipolytic organisms, when grown on a solid medium in which was dispersed a suitable fat or oil, produce a lipase which causes the disappearance of the fat from the immediate vicinity of the growth. Waksman and Davison (1926) emphasized the use of the changes produced in certain indicators by the freed fatty acids to detect fat hydrolysis through the action of organisms.

The detection of fat hydrolysis by bacteria was investigated by Turner (1927). He used a medium composed of 1000 ml. of sugar-free meat digest fluid, 5 grams of di-basic sodium phosphate and 30 grams of agar, the reaction of the medium being adjusted to a final pH of 7.6. After autoclaving, Nile-blue sulphate was added and the medium tubed and heated in an Arnold. A sterile fat emulsion was added to the melted and cooled medium. Turner (1929) compared the relative merits of various methods for the determination of fat hydrolysis by microorganisms and concluded that the Nile-blue sulphate medium gave remarkable sharpness of differentiation. He used various simple tri-glycerides

in the medium and found that tri-butylin inhibited growth of the two test cultures. Nile-blue sulphate, in concentrations of 1:8000, inhibited the growth of a number of organisms; the flooding of plates with Nile-blue sulphate solution after incubation was suggested for certain purposes. Turner considered that a good differential plating medium, without dye, for the detection of lipolytic bacteria should result from either an emulsion of fat made up of short-chained tri-glycerides or from the use of bile in the medium along with an oil like cottonseed oil.

Kaufmann and Lehmann (1926), studied the action of various stains, including Nile-blue sulphate, on different organic acids, esters and other materials. They noted the striking effect of Nile-blue sulphate on unsaturated fatty acids and unsaturated tri-glycerides but found that certain compounds without double bonds were also stained.

Rettie (1931) considered that Nile-blue sulphate contains two coloring reagents, the blue which is soluble in the fatty acids and the pink which is soluble in the fats; the latter he called Nile-pink.

Turner's technique was somewhat modified by Hussong (1932). Hussong used beef infusion agar with a pH of 6.8 to 7.0. To this he added Nile-blue sulphate (as an alcoholic solution) in the ratio of 1:10,000 and fat in the proportion of 1:200. The fat was emulsified in 0.5 per cent agar and was sterilized before it was added to the medium. Cultures were streaked on the surface of the medium. A change in the color of the fat globules was accepted as evidence of hydrolysis.

Berry (1933) worked with the test originated by Carnot and Mauban (1918) for the detection of microbial lipase. The organisms were grown on a solid medium having fat dispersed in it, and when good growth had occurred the plates were flooded with saturated copper sulphate solution. If hydrolysis had taken place, the freed fatty acids reacted with the copper sulphate to give an insoluble blue soap.

STATEMENT OF PROBLEM

The work herein reported was undertaken (a) to determine the action of Nile-blue sulphate on various simple tri-glycerides and the corresponding fatty acids and on some natural and hydro-

genated fats, (b) to study certain factors affecting the detection of lipolysis by bacteria, using Nile-blue sulphate and (c) to determine the susceptibility of various simple tri-glycerides and natural and hydrogenated fats to the action of lipolytic bacteria. All of the trials were carried out in agar media.

GENERAL ACTION OF NILE-BLUE SULPHATE ON FAT

The general action of Nile-blue sulphate in the staining of fat was shown by the investigations of Thorpe (1907) which followed observations by Smith and White that certain blue coloring materials of the oxazine series, when used as a stain for sections containing neutral fat, possessed the property of coloring fatty matter red, while protein matter was stained blue. Smith and White had noted that the red stain could be extracted by xylene. Thorpe found that the red coloring matter could not be secured from the solid dye, but that it was apparently formed when the dye was dissolved in water. By treating the dye dissolved in water with sulphuric acid the formation of the red dye readily took place and it could be extracted with xylene; the production of the red dye was increased by heating the mixture on a water bath. In the preparation of certain fat stains the dye is boiled with acid to intensify the staining of fat. Nile-blue sulphate is a sulphate of diethylaminophenonaphthoxazine and the red dye the corresponding oxazone.

METHODS

The medium ordinarily used for studying the action of Nile-blue sulphate on simple tri-glycerides, fatty acids and natural and hydrogenated fats, and also for investigating the action of bacteria on simple tri-glycerides and fats, was beef-infusion agar adjusted to a pH of 6.8 to 7.0. A 0.1 per cent aqueous solution of Nile-blue sulphate was added to the agar, in the proportion of 10 to 100 ml. of medium, and the agar then put into tubes or flasks and sterilized. Lower concentrations of Nile-blue sulphate were also used with satisfactory results, but the colors were less intense.

The emulsions of natural and hydrogenated fats were prepared as follows: The fat to be used was filtered with a hot water funnel

and added to a melted 0.5 per cent agar solution in the proportion of 10 ml. of fat to 90 ml. of the solution. The mixture was sterilized at 15 pounds for twenty-five minutes, allowed to cool until it was solidified and then vigorously shaken to secure an emulsion of the fat. The fat emulsion was stored in this condition and just before use was heated to a temperature that would give a soft jelly-like mass which could be easily transferred with a pipette.

When plates were to be poured, agar containing Nile-blue sulphate was melted and the fat emulsion added to the hot agar in the proportion of 1 ml. of the emulsion to 20 ml. of the agar. After the dye had been added to the agar the medium was allowed to remain hot for a few minutes before it was poured.

The liquid tri-glycerides and fatty acids were dispersed in agar containing Nile-blue sulphate in the same general manner as the natural and hydrogenated fats, excepting that smaller quantities were used because of the cost. The tri-glycerides and the fatty acids which are solid at 21°C. were dispersed as follows: Agar containing Nile-blue sulphate was added to the plates and kept hot over a low Bunsen flame, while a small amount of the solid tri-glyceride or fatty acid was added and vigorously stirred into it. The agitation was continued until the fat or fatty acid had solidified in small globules or masses.

In the study of the effect of various bacteria on the simple tri-glycerides and natural and hydrogenated fats, the plates poured with the materials were left at room temperature until the surface of the medium was dry (at least twelve hours) to prevent an abnormal spreading of the bacterial colonies. Several organisms were inoculated on each plate, using a small loop-full of a forty-eight-hour litmus milk culture of each organism to be studied. The plates were inverted, incubated at 21°C. and examined frequently for evidence of lipolysis.

All the examinations for color changes and for disappearance of the globules were made with a hand lens or a wide field binocular.

The tri-olein was prepared by Dr. Fraenkel and Dr. Landau, Berlin, while the other simple tri-glycerides were secured from the Eastman Kodak Company. Some of the fatty acids were ob-

tained from the Eastman Kodak Company, and the others from Merck and Company. The natural and hydrogenated fats were secured locally.

RESULTS OBTAINED

Action of Nile-blue sulphate on various simple tri-glycerides, fatty acids and natural and hydrogenated fats

The action of Nile-blue sulphate on various simple tri-glycerides, fatty acids and natural and hydrogenated fats was studied by pouring plates with agar containing Nile-blue sulphate, as outlined under Methods. The plates were held at approximately 21°C. and examined frequently. In a number of trials, an agar containing no added nutrients was employed in addition to beef-infusion agar; the results were the same with the two media.

Action on simple tri-glycerides. The simple tri-glycerides used were tri-acetin, tri-propionin, tri-butylin, tri-caproin, tri-caprylin, tri-caprin, tri-laurin, tri-myristin, tri-palmitin, tri-olein and tri-stearin.

Tri-acetin, in the concentrations used, was completely soluble in the medium. The globules of tri-propionin, tri-butylin, tri-caproin and tri-caprylin were bright red in color. The dispersed masses of tri-caprin, tri-laurin, tri-myristin, tri-palmitin and tri-stearin were solid and colored red to a degree which decreased rapidly with the increase in the melting points, until very little red color was present. A rather uniform distribution of fairly round solid globules was obtained with tri-caprin and tri-laurin, while with tri-myristin, tri-palmitin and tri-stearin a dispersion of somewhat flaky, irregularly shaped masses was secured; the color of these irregular masses ranged from a faintly purplish red to a faintly reddish purple and, occasionally, to a bluish white. The dispersed globules of tri-olein were very uniformly bright red and regular in size.

Action on fatty acids. The fatty acids employed were acetic, propionic, butyric, caproic, caprylic, capric, lauric, myristic, palmitic, oleic and stearic.

Acetic, propionic and butyric acids were soluble in the media in the concentrations used and, with the occasional exception of butyric acid, they did not affect the appearance; butyric acid sometimes caused a turbidity in the media. The dispersed globules of caproic and caprylic acids became distinctly blue and seemed to absorb the dye from the surrounding medium uniformly. The dispersed, solid, disc-like globules of capric and lauric acids ranged in color from a blue that was darker than the surrounding medium to one that was much lighter. The masses of myristic, palmitic and stearic acids were very lightly and not uniformly stained, the stainability seeming to depend on the shape and size of the mass. The globules of oleic acid were always a clear blue that was much darker than the surrounding media.

Action on natural and hydrogenated fats. The natural fats used were beef tallow, butter fat, cocoanut oil, corn oil, cottonseed oil, lard, linseed oil and olive oil. Dispersed portions of all of these materials became distinctly red when added to media containing Nile-blue sulphate. With linseed oil and olive oil the globules were smaller than with the other materials and, presumably because of the size, did not appear as red.

Cottonseed oil and lard that had been commercially hydrogenated gave essentially the same changes with Nile-blue sulphate as the unhydrogenated fats.

Rate of color change when various materials were added to agar containing Nile-blue sulphate. When simple tri-glycerides, insoluble fatty acids or natural fats were added to agar containing Nile-blue sulphate the usual color change occurred very quickly; the time required seemed to be decreased by temperatures considerably above room temperature. When an unemulsified fat was added to hot agar containing Nile-blue sulphate and the agar shaken vigorously for thirty seconds the fat was conspicuously pink by the time it had collected at the surface. Fat which had been sterilized a number of times showed a less rapid and less definite color change with Nile-blue sulphate than fat which had been sterilized only once.

Factors affecting the detection of lipolysis by bacteria, using Nile-blue sulphate

Comparison of the disappearance of globules of simple tri-glycerides and the color changes in them by Nile-blue sulphate. A comparison of the disappearance of globules of simple tri-glycerides and the color changes in them by Nile-blue sulphate for the detection of lipolysis by bacteria was made with 100 miscellaneous cultures. The organisms were isolated from a variety of sources; most of them were lipolytic, but a few non-lipolytic organisms were included as controls. Comparisons could be made best with tri-caproin and tri-caprylin, since the acids of the lower tri-glycerides were completely soluble in the medium in the concentrations used, and the acids of the higher tri-glycerides were insoluble, or only very slightly soluble, but comparisons were also possible with tri-caprin.

With tri-caproin and tri-caprylin, hydrolysis was detected more readily by the disappearance of globules than by the development of a blue color, while with tri-caprin the reverse was true. It appears that this variation is due to the solubility of the acid freed by hydrolysis; if the acid is comparatively soluble its rapid diffusion results in less blue color than if the acid is only very slightly soluble. The best agreement between the two changes was secured with tri-caprin; capric acid was apparently sufficiently soluble so that the disappearance of globules was usually detected, and at the same time sufficiently insoluble so that the blue color was observed. With certain tri-glycerides there was a variation in the results obtained with different organisms so that some organisms caused a conspicuous disappearance of the globules while others caused a conspicuous development of a blue color. Presumably, variations in the action of the different organisms on the primary hydrolytic products influence this reaction.

It should be noted that when natural fats were dispersed in the agar there was no indication of a disappearance of the globules and, for this reason, the color change was always the basis on which the action of the organisms was determined.

Influence of the method of dispersing the tri-glycerides in the medium. The influence of the method of dispersing the tri-

glycerides in the medium on the detection of lipolysis by bacteria was studied with tri-butylin, tri-caproin, tri-caprylin and tri-olein, the simple tri-glycerides that appear to be most useful in investigating the action of bacteria. These materials were added to melted agar as an emulsion in 0.5 per cent agar and also directly. In each case the medium was then shaken and poured into plates. When the tri-glycerides were emulsified in the agar a more uniform dispersion in the plate was secured than when they were added directly. The comparisons of the action of bacteria on the tri-glycerides dispersed with the two methods showed that the same results were secured with the two procedures.

With the natural fats it was possible to prepare reasonably satisfactory plates by adding the fat directly to the melted agar containing the Nile-blue sulphate, but the dispersion was not as uniform as when the fat was emulsified in 0.5 per cent agar, and there was a tendency for the globules to rise to the surface of the medium and to form masses which interfered with the growth of the organisms.

Influence of the pH of the medium. The effect of the pH on the detection of lipolysis by bacteria in beef-infusion agar containing Nile-blue sulphate was studied with tri-butylin, tri-olein and cottonseed oil, using 26 cultures of bacteria that had been isolated from various sources; most of the organisms were lipolytic. The pH values used were 5.3, 6.7 and 7.8. With tri-olein and cottonseed oil an organism which hydrolyzed at one pH also hydrolyzed at the others, although with many of the organisms the most alkaline reaction seemed to favor hydrolysis, as evidenced by the rate at which the color change appeared. With tri-butylin at a pH of 5.3 the development of colonies was much slower than at a pH of 6.7 or 7.8, and some of the organisms were entirely inhibited; however, if growth occurred, the organisms which hydrolyzed at a pH of 6.7 or 7.8 also eventually hydrolyzed at a pH of 5.3.

Susceptibility of various simple tri-glycerides and natural and hydrogenated fats to the action of lipolytic bacteria

The action of bacteria on various simple tri-glycerides and natural and hydrogenated fats, as shown by Nile-blue sulphate,

was studied with a considerable number of cultures. Most of the cultures were isolated from various sources, but a few were secured from culture collections. Many of the organisms isolated had hydrolyzed fat on the plates used for isolation, but a number which had failed to hydrolyze were also included. The action of each organism was tried three or four times on each simple tri-glyceride and from two to four times on each natural or hydrogenated fat.

TABLE 1

The hydrolysis of some simple tri-glycerides by 119 cultures of bacteria as indicated by Nile-blue sulphate

TRI-GLYCERIDE	PER CENT OF CULTURES SHOWING		
	Hydrolysis	No hydrolysis	Questionable hydrolysis
Tri-propionin	98.3	1 7	0 0
Tri-butylin	78 2	17 6	4 2
Tri-n-valerin	68 0	28 0	4 0
Tri-iso-valerin	50 0	40 0	10 0
Tri-caproin	53 8	44 5	1 7
Tri-heptylin	36 0	48 0	16.0
Tri-caprylin	56 2	39 5	4 3
Tri-caprin	27 7	63 0	9 3
Tri-laurin	26 5	70 0	3.5
Tri-myristin	9 2	87.6	3 2
Tri-palmitin	1 7	98 3	0 0
Tri-olein	74.8	19.3	5.9
Tri-stearin	0 0	100 0	0.0

Action on simple tri-glycerides. The results obtained on the simple tri-glycerides with 119 cultures are summarized in table 1. From the data it is evident that, as determined by Nile-blue sulphate, the hydrolysis of the simple tri-glycerides of the saturated fatty acids became more difficult as the molecular weight increased. Tri-propionin was hydrolyzed by 98.3 per cent of the cultures and tri-stearin by none of them. A considerably larger percentage of the organisms hydrolyzed tri-propionin than any of the other simple tri-glycerides. Tri-olein was hydrolyzed by 74.8 per cent of the cultures, a percentage that is only slightly

lower than the percentage that hydrolyzed tri-butylin; the detailed data show that there were a few cultures which hydrolyzed one of these tri-glycerides but not the other. It is of interest to note that the tri-glycerides of the fatty acids containing an uneven number of carbon atoms were hydrolyzed by considerable percentages of the organisms; however, tri-heptylin was hydrolyzed by a smaller percentage of the organisms than tri-caproin or tri-caprylin, and tri-iso-valerin by a smaller percentage than tri-n-valerin.

TABLE 2

The hydrolysis of some natural and hydrogenated fats by 92 cultures of bacteria as indicated by Nile-blue sulphate

NATURAL FAT OR OIL	PER CENT OF CULTURES SHOWING		
	Hydrolysis	No hydrolysis	Questionable hydrolysis
Beef fat	83.7	15.2	1.1
Butter fat	80.4	18.5	1.1
Cocoanut oil	77.2	21.7	1.1
Corn oil	84.8	15.2	0.0
Cottonseed oil	78.3	20.6	1.1
Lard	86.0	14.0	0.0
Linseed oil	83.7	15.2	1.1
Olive oil	82.6	16.3	1.1
Hydrogenated cottonseed oil	79.2	20.8	0.0
Hydrogenated lard	79.2	20.8	0.0

Action on natural and hydrogenated fats. The results secured on natural and hydrogenated fats with 92 cultures are summarized in table 2. The data indicate that, as shown by Nile-blue sulphate, there was little variation in the percentage of the organisms that hydrolyzed the different materials, the values ranging from 77.2 to 86.0 per cent; cocoanut oil was hydrolyzed by the smallest percentage of the organisms and lard by the largest percentage. The detailed data for the individual organisms indicate that when an organism hydrolyzed one of the fats it also hydrolyzed most of the others. The hydrogenation of cottonseed oil and lard did not significantly influence the action of the bacteria on them.

DISCUSSION OF RESULTS

The difference between the color change produced by Nile-blue sulphate in certain fatty acids and that produced in certain simple tri-glycerides and in natural fats forms the basis of a convenient test for detecting the ability of organisms to hydrolyze fat when dispersed in an agar medium. While with natural fats the color change is the principal factor to be taken into consideration, with certain of the simple tri-glycerides the disappearance of the globules without the formation of a blue color may occur because of the solubility of the acids.

The presence of the indicator in the medium is convenient because it permits repeated observations on the action of organisms. It should be recognized, however, that Nile-blue sulphate may inhibit the growth of some organisms under certain conditions.

The variations in the action of bacteria on the simple tri-glycerides is much greater than the variations in the action on natural fats. This suggests that if differences in the lipolytic powers of bacteria are to be used in classification studies they should be investigated with simple tri-glycerides rather than mixed tri-glycerides.

SUMMARY

1. When dispersed in agar containing Nile-blue sulphate, tri-propionin, tri-butylin, tri-caproin, tri-caprylin and tri-olein were colored bright red, and tri-caprin, tri-laurin, tri-myristin, tri-palmitin and tri-stearin were colored red to a degree which decreased rapidly with the increase in the melting points, until very little red color was present. Under similar conditions, caproic, caprylic and oleic acids were colored uniformly blue, capric and lauric acids varied in the intensity of the blue, while myristic, palmitic and stearic acids absorbed very little of the blue; the blue color was especially intense with oleic acid.

2. When dispersed in agar containing Nile-blue sulphate, beef tallow, butter fat, cocoanut oil, corn oil, cottonseed oil, lard, linseed oil and olive oil were colored bright red; linseed oil and

olive oil did not appear as red as the others, due presumably to the small size of the globules.

3. With tri-caproin and tri-caprylin, hydrolysis by bacteria was more readily detected by the disappearance of the globules than by the color change with Nile-blue sulphate. With natural fats the disappearance of the globules was not clearly evident, and the results were based on the color change.

4. As shown by Nile-blue sulphate, the hydrolysis by bacteria of various simple tri-glycerides and natural fats was not appreciably affected by (a) the manner in which the materials were dispersed or (b) the pH of the medium within the limits used; however, the more alkaline reaction favored the growth of the lipolytic bacteria studied.

5. Tri-propionin was more easily hydrolyzed by bacteria than various other simple tri-glycerides or natural and hydrogenated fats.

6. In general, the hydrolysis by bacteria of simple tri-glycerides of the saturated fatty acids was more difficult as the molecular weight increased. Tri-olein was comparatively easily hydrolyzed.

7. In general, each of the organisms used had much the same action on various natural and hydrogenated fats.

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The American Type Culture Collection

IN 1925 a collection of bacteria, maintained for 11 years by the American Museum of Natural History in New York City, and after 1922 at the Army Medical Museum in Washington, D. C., was established in the John McCormick Institute for Infectious Diseases in Chicago as the American Type Culture Collection. It is administered by a committee representing the Society of American Bacteriologists, the American Phytopathological Society, the Society of Pathologists and Bacteriologists, the Society of American Zoologists, and the McCormick Institute.

The collection maintained at the McCormick Institute contains at the present time about 1300 cultures representing all branches of bacteriology. In addition, connections have been established with other laboratories at home and abroad which have made a large number and variety of bacteria, yeasts, and fungi available for distribution.

In the 9 years that the collection has been under the present management over 34,000 cultures have been distributed. The greater part of these have gone to institutions mostly for teaching purposes, but the calls for cultures for industrial purposes have increased materially. The collection has been able to be of additional service by obtaining cultures for special purposes from laboratories in this country and Europe.

The collection was established on a grant from the General Education Board, but since the expiration of this fund it has been supported by the income from the sale of cultures supplemented by a small grant from the Society of American Bacteriologists and a few contributions from industrial companies. Under these conditions it has been necessary to exercise rigid economy in the management of the collection, but this has been done without any serious impairment of its efficiency and only a small reduction in the number of available cultures.

With the removal of substantial support from outside sources, it became necessary to increase the price of cultures and this has, as would be expected, caused a falling off in the number of cultures sold. The depressed business conditions have also affected the sale of cultures adversely, but in spite of these unfavorable conditions nearly 3000 cultures were distributed in the past year, and the committee was able to meet all obligations and finish the year with a small balance.

It is the aim of the committee to make available to the investigator and teacher authentic cultures of the greatest range possible of bacteria, yeasts, and fungi. Without the aid of funds aside from those coming from the sale of cultures, further expansion of the collection is impossible. When funds are available protozoa will be added.

Since, under present conditions, it seems possible to provide this service only through the sale of cultures, it is hoped that those interested will call the attention of their friends and correspondents to the service offered by the collection, which may be addressed at 637 South Wood Street, Chicago, Illinois.

L. A. ROGERS.

TYPES OF LIPOLYSIS BROUGHT ABOUT BY BACTERIA, AS SHOWN BY NILE-BLUE SULPHATE¹

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The addition of Nile-blue sulphate to a nutrient agar containing dispersed fat provides a convenient method for the detection of bacteria that hydrolyze the fat. The evidence of lipolysis obtained with this procedure requires interpretation, especially from the standpoint of the significance of the organisms in dairy products. The work herein reported deals with (a) the types of lipolytic action shown by various bacteria on agar containing dispersed fat and Nile-blue sulphate, (b) the relationship between the lipolytic and proteolytic activities of organisms and (c) the effect of lipolytic organisms on unsalted butter held at temperatures favorable for growth.

CULTURES USED

The cultures studied were isolated from various sources such as water, air, dairy-plant equipment and dairy products. They included 10 cultures of *Achromobacter Connii* (Chester) Bergey *et al.* (1930) and 7 cultures closely related to this species; 11 cultures of *Achromobacter lipolyticum* (Huss, 1908) Bergey *et al.* (1930); 3 cultures of *Bacterium viscosum* (Adametz, 1891) (Hammer, 1928); 6 cultures of *Pseudomonas acidiconcoquens* (Collins, 1933); 19 cultures of *Pseudomonas fluorescens* (Flügge) (Migula, 1900); 30 cultures closely related to *Pseudomonas fluorescens* and classified as varieties of this species (Collins, 1933); 19 cultures of *Pseudomonas fragi* (Eichholz) Hussong (1932); 1 culture of

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Pseudomonas mucidolens Anderson (1930); Levine and Anderson (1932); 1 culture of *Pseudomonas myxogenes* Fuhrmann (1907); 2 cultures of *Pseudomonas schuylkilliensis* (Wright) Chester (1901) and 2 closely related cultures; 3 cultures of *Pseudomonas synxantha* (Ehrenberg) Hammer (1928), according to the description published by Hammer (1915); and 1 unidentified culture belonging to the genus *Serratia*. Thirty-one cultures of Gram-negative, non-spore forming rods and 13 cultures of micrococci were not identified.

METHODS

The medium used for studying the action of bacteria on fat, in the presence of Nile-blue sulphate, was beef-infusion agar adjusted to a pH of 6.8 to 7.0. An 0.1 per cent aqueous solution of Nile-blue sulphate was added to the agar in the proportion of 10 ml. to 100 ml. of medium. The agar was then put into tubes and sterilized.

The fat to be used was filtered with a hot water funnel and added to a melted 0.5 per cent agar solution in the proportion of 10 ml. of fat to 90 ml. of the solution. The mixture was sterilized at 15 pounds for twenty-five minutes, cooled until it was solidified and then vigorously shaken to secure an emulsion of the fat. Just before use, the fat emulsion was heated to a temperature that would give a soft jelly-like mass which could be easily transferred with a pipette.

When the plates were to be poured, the agar containing Nile-blue sulphate was melted and the fat emulsion added to the hot agar in the proportion of 1 ml. of the emulsion to 20 ml. of the agar. The agar containing the fat was allowed to remain hot for a few minutes before it was poured, since this intensified the red color of the fat.

RESULTS OBTAINED

Types of lipolytic action shown by bacteria on agar containing Nile-blue sulphate and dispersed fat

The types of bacterial action on fat dispersed in agar, as shown by Nile-blue sulphate, were studied from the standpoint of the

changes occurring in the globules beneath and around the colonies. Large colonies were secured by touching the surface of the agar with a small loop that had been put into a milk culture of the organism to be studied; commonly a plate was used for a number of organisms. The plates were incubated at 21°C. and observations made at various times, using a hand lens or a wide-field binocular. When practically all the globules beneath the colonies became blue the lipolysis was considered uniform, while when some of the globules became blue and some remained red the lipolysis was considered non-uniform. Both uniform and non-uniform lipolysis were divided further on the basis of the amount of diffusion of the lipase produced. When there was no color change in the globules around the colonies the diffusion was regarded as slight, while when there was a color change extending out around the colonies the diffusion was regarded as conspicuous; the conspicuous diffusion was recorded by one, two or three plus signs, depending on the width of the zone of blue globules around the colonies.

The fat dispersed in the agar was usually butter fat. Comparisons of butter fat with other natural fats, especially beef tallow, indicated that, as shown by Nile-blue sulphate, an organism brought about the same general type of change in various fats.

The types of lipolysis produced by bacteria were investigated with 159 cultures isolated from various sources (see page 487). Each culture was studied from two to six times and the results for each culture were very consistent. A summary of the data secured is presented in table 1.

The results show that 102 (64.2 per cent) of the 159 cultures brought about uniform lipolysis, while the other 57 (35.8 per cent) brought about non-uniform lipolysis. Fifteen (14.7 per cent) of the 102 cultures giving uniform lipolysis showed only slight lipase diffusion, while 87 (85.3 per cent) showed conspicuous lipase diffusion. With 62 (71.3 per cent) of the 87 cultures there was enough lipase diffusion to form small zones of hydrolyzed fat about the colonies (+), with 20 (23.0 per cent) fairly large zones were formed (++), while with 5 (5.7 per cent) there was enough diffusion to form zones wider than the diameter

of the colonies themselves (+++). With the 57 cultures which gave non-uniform lipolysis 36 (63.2 per cent) showed slight lipase diffusion, and the remaining 21 (36.8 per cent) showed conspicuous diffusion. Of the cultures which showed conspicuous diffusion 15 (71.4 per cent) produced small zones of hydrolyzed fat

TABLE 1

Types of lipolytic action of 159 cultures of lipolytic bacteria on fat dispersed in agar, as shown by Nile-blue sulphate

SPECIES, VARIETY OR MORPHOLOGIC TYPE	TOTAL NUMBER OF CULTURES	LIPOLYTIC ACTION UNIFORM				LIPOLYTIC ACTION NON-UNIFORM			
		Lipase diffusion				Lipase diffusion			
		Slight	Conspicuous			Slight	Conspicuous		
			+	++	+++		+	++	+++
<i>A. Connii</i> and closely related types.....	17	6	9			2			
<i>A. lipolyticum</i>	11		8	2		1			
<i>Bact. viscosum</i>	3		3						
Gram negative, non-spore forming rods	31	1	10	2		13	3	2	
Micrococci.....	13	2	3			7		1	
<i>Ps. acidiconcoquens</i>	6	3	1			2			
<i>Ps. fluorescens</i>	19		12	4	2		1		
<i>Ps. fluorescens</i> varieties.....	30	1	9	7	3	2	6	2	
<i>Ps. fragi</i>	19	1	4	3		5	5	1	
<i>Ps. mucidolens</i>	1			1					
<i>Ps. myxogenes</i>	1			1					
<i>Ps. schuylkilliensis</i> and closely related types.....	4		2			2			
<i>Ps. synxantha</i>	3	1	1			1			
<i>Serratia</i> species.....	1					1			
Totals.....	159	15	62	20	5	36	15	6	

about the colonies (+), while 6 (28.6 per cent) formed fairly large zones (++).

From table 1 it is evident that the cultures within a species or group showed a considerable variation in the types of lipolysis produced, both from the standpoint of whether the lipolysis was uniform or non-uniform and from the standpoint of the diffusion

of the enzyme. Although the numbers of cultures of the various species studied are very different, the data suggest that variations in the type of lipolysis are greater with some species or groups than with others.

The species that were most constant in their type of lipolytic actions were *A. Connii* and closely related types, *A. lipolyticum*, *Bact. viscosum* and *Ps. fluorescens*. *Ps. acidiconcoquens* was also quite constant in that with 5 of the 6 cultures there was slight diffusion of the enzyme, but 4 of the cultures produced uniform lipolysis and 2 non-uniform lipolysis. The *Ps. fluorescens* varieties and *Ps. fragi* varied a great deal in the type of lipolysis produced, and the same was true of the miscellaneous groups of unidentified, Gram-negative, non-spore forming rods and of micrococci. It should be noted that with most of the cultures of *A. lipolyticum*, *Ps. fluorescens* and *Ps. fluorescens* varieties conspicuous lipase diffusion occurred.

Relationship of lipolytic and proteolytic activities of lipolytic bacteria

One of the important points with reference to the cultures of lipolytic bacteria studied is that many of them very evidently proteolyze milk, as shown by the appearance of litmus-milk cultures. Of the 159 cultures investigated, 113 (71.1 per cent) definitely proteolyzed milk, as indicated by the general changes in litmus milk, while 46 (28.9 per cent) did not; certain of the latter cultures presumably would have shown protein decomposition if the fermented milk had been examined chemically. With the 113 cultures showing conspicuous proteolysis there was a variation in the rate and extent of the change, 82 (72.6 per cent) of the cultures being very actively proteolytic while 31 (27.4 per cent) gave a slow or partial proteolysis. Among the active proteolyzers *A. lipolyticum* and the *Pseudomonas* cultures, with the exception of *Ps. fragi*, were especially important. *Ps. fragi* is an organism which shows striking variations and some of the cultures produced a slow proteolysis while the others produced no proteolysis, as shown by the appearance of milk cultures. In general, the cultures of *Ps. fragi* which showed pronounced pro-

teolysis were the ones that gave uniform lipolysis and conspicuous lipase diffusion. Cultures of *A. lipolyticum*, *Ps. fluorescens*, *Ps. mucidolens*, and some of the *Ps. fluorescens* varieties, were actively proteolytic and actively lipolytic. *Ps. acidiconcoquens* was very actively proteolytic but not actively lipolytic.

Action of lipolytic bacteria on butter

The general action of lipolytic bacteria on butter was studied with 80 of the 159 cultures. The usual procedure was to inoculate about 500 ml. of sterilized cream, that had been well cooled to solidify the fat, with 3 ml. of a two-day milk culture of one of the organisms and churn it in a glass jar; the butter was washed with sterile water, worked in a sterile dish with a sterile paddle and then packed in sterile glass jars. The butter was unsalted and was held at 21°C., the object being to make conditions as favorable as possible for the growth of the organisms. The numbers of bacteria per milliliter of the fresh butter varied from about 50 to about 500,000 per milliliter. The butter was examined for defects from time to time and carefully compared with a lot of butter made from the uninoculated cream. When no defect was detected within three weeks, the organism added was considered to have no effect on butter. The production of rancidity was recorded as rapid when it occurred within three days, as medium when it occurred in from four days to one week and as slow when more than one week was required. The action of some of the cultures on butter was studied only once, but the action of a considerable number was investigated several times; when an individual culture was used more than once the results obtained with it were consistent. Table 2 presents the results of the trials with the organisms and also a summary of the lipolytic action of the organisms.

The data show that, of the 80 cultures which hydrolyzed fat dispersed in beef-infusion agar, 60 (75.0 per cent) produced rancidity in butter under the conditions used; 26 (32.5 per cent) produced it within three days while 34 (42.5 per cent) required a longer time. In general, the species or groups which produced rancidity rapidly commonly produced uniform lipolysis and

the diffusion of the enzyme was conspicuous. The data for the individual cultures indicate that, of the 26 cultures which caused rancidity rapidly, 22 (84.6 per cent) produced uniform lipolysis with conspicuous lipase diffusion, while of the 34 cultures which

TABLE 2

Relationship between lipolytic action of 80 cultures of bacteria, as shown by Nile-blue sulphate, and the production of rancidity in butter

SPECIES, VARIETY OR MORPHOLOGIC TYPE	NUMBER OF CULTURES	LIPOLYTIC ACTION				PRODUCTION OF RANCIDITY		
		Uniform		Non-uniform		Rapid	Medium to slow	None
		Lipase diffusion		Lipase diffusion				
		Slight	Conspicuous	Slight	Conspicuous			
<i>A. Connii</i> and closely related types.....	4	3	1				1	3
<i>A. lipolyticum</i>	8		8			8		
<i>Bact. viscosum</i>	2		2				2	
Gram negative, non-spore forming rods ..	10	1	3	4	2	1	3	6
Micrococci.....	4	2		2				4
<i>Ps. acidiconcoquens</i> ..	6	3	1	2			4	2
<i>Ps. fluorescens</i> ..	15		15			11	3	1
<i>Ps. fluorescens</i> varieties.	12	1	4	2	5	2	9	1
<i>Ps. fragi</i>	10		2	3	5	2	6	2
<i>Ps. mucidolens</i> ..	1		1			1		
<i>Ps. myrogenes</i>	1		1			1		
<i>Ps. schuykilliensis</i> and closely related types ..	4		3		1		4	
<i>Ps. synxantha</i> ..	2		2				1	1
<i>Serratia</i> species ..	1				1		1	
Totals..	80	10	43	13	14	26	34	20

produced medium to slow rancidity 13 (38.2 per cent) produced uniform lipolysis with conspicuous lipase diffusion.

Some of the cultures of the *Ps. fluorescens* varieties and also some of the *Ps. fragi* cultures produced rancidity slowly in butter but did not give uniform lipolysis. Among the cultures which did not cause rancidity in butter, *A. connii* and closely related

types, Gram-negative, non-spore forming rods, and micrococci were especially important. The first of these groups generally produced uniform lipolysis while the others did not.

Some of the cultures which produced rancidity in butter also produced another defect along with the rancidity; several cultures produced rancidity and cheesiness and several produced rancidity and a "May Apple" odor.² The 20 cultures which failed to produce rancidity had various effects on the flavor of the butter; 3 produced a slightly putrefactive condition, 1 produced a "May Apple" odor,² 1 produced a musty condition, while with 15 there were indefinite or no flavor changes.

DISCUSSION OF RESULTS

The results obtained indicate that, as shown by Nile-blue sulphate, there is considerable variation in the types of lipolysis produced by bacteria on fat dispersed in agar. These variations may prove to be useful in the study of lipolytic bacteria but, since the organisms belonging to a species also show considerable variation, the interpretation of the type of lipolysis an organism produces must be carefully considered.

The frequency with which lipolytic bacteria are also actively proteolytic is a point of considerable interest. This should be useful in connection with the isolation of lipolytic organisms from dairy products that have undergone changes in the fat. It also suggests that the lipolytic bacteria can be expected to produce changes in the protein of dairy products; this protein decomposition may have a significant effect on the flavor and odor.

The variations in the lipolytic abilities of organisms is evident from the action of the 80 cultures studied for their effect on butter. In general, the organisms which show uniform lipolysis on fat dispersed in agar with conspicuous lipase diffusion are more likely to produce rancidity in butter than those which are less actively lipolytic.

² The odor of the flower and fruit of the May Apple or mandrake (*Podophyllum peltatum*).

SUMMARY

1. Of the 159 lipolytic cultures studied, 102 (64.2 per cent) brought about uniform hydrolysis of fat dispersed in agar, as shown by Nile-blue sulphate, and 57 (35.8 per cent) brought about non-uniform hydrolysis. With both groups there was considerable variation in the extent of the diffusion of lipase from the area of growth, some of the cultures showing slight diffusion while others showed conspicuous diffusion.

2. Although each culture was consistent in the type of lipolytic action, as shown in from two to six trials per culture, the cultures within a species or group showed considerable variation.

3. Among the organisms studied, *A. Connii* and closely related types, *A. lipolyticum*, *Bact. viscosum* and *Ps. fluorescens* were the most constant in the type of lipolysis produced.

4. Of the 159 lipolytic cultures studied, 113 (71.1 per cent) very evidently proteolyzed milk, as shown by the appearance of litmus milk cultures; 82 (72.6 per cent) of the 113 cultures were very actively proteolytic while 31 (27.4 per cent) produced slow or partial proteolysis.

5. Eighty of the cultures which hydrolyzed fat dispersed in beef infusion agar, as shown by Nile-blue sulphate, when studied for their action on unsalted butter held at 21°C., and 60 (75.0 per cent) produced rancidity in the butter. In general, the species or groups of organisms which produced rancidity rapidly commonly showed uniform lipolysis and the lipase diffusion was conspicuous.

6. Certain of the cultures of lipolytic bacteria which produced rancidity in butter produced another defect along with the rancidity. Some of the cultures which failed to produce rancidity caused various types of defects, while others produced indefinite or no flavor changes.

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A TAXONOMIC STUDY OF "CLOSTRIDIUM PUTRIFICUM" AND ITS ESTABLISHMENT AS A DEFINITE ENTITY—CLOSTRIDIUM LENTOPUTRESCENS, NOV. SPEC.¹

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HISTORY

The name "*Cl. putrificum*"² was applied by Sturges and Rettger to a putrefactive anaerobe which was first isolated by them in 1919, and studied in greater detail by Reddish and Rettger (1921, 1922, 1923). These authors believed their organism to be identical with Bienstock's *B. putrificus* and, largely through the influence of their studies, the species "*Cl. putrificum*" has been recognized by American bacteriologists as an established entity.

Quite recently Cunningham (1932) revived the issue regarding the identity of the species of anaerobe which Bienstock characterized. Through a reinterpretation of Bienstock's publications, and the correlation of the characteristics of a proteolytic anaerobe which he had just isolated, Cunningham maintained that he possesses strains which have more in common with Bienstock's organism than do those of Rettger and his associates. He suggested that the last-named authors were dealing with *B. cochlearius*, and not Bienstock's *B. putrificus*, although he appreciated the fact that *B. cochlearius* and "*Cl. putrificum*" differ in their morphology and their ability to attack milk and to liquefy gelatin. He regarded the observations of Hall (1922), Fildes

¹ This paper covers in part the work submitted to the Graduate School of Yale University by the senior author as part requirement for the Degree of Doctor of Philosophy.

² It will be convenient to designate the organism regarded by Reddish and Rettger as *Cl. putrificum* Bienstock in quotation marks throughout this report.

(1929) and Cunningham (1931) as supporting his tentative conclusions.

It appeared to the present authors that when two anaerobic species differ in such fundamental properties as morphology and ability to attack protein media, it would be inconsistent with the principles of modern classification to include them under one specific name or species. The so-called "*Cl. putrificum*" has exhibited constant cultural characteristics, and we can see no reason for regarding it as anything other than a definite entity. This should be all the more apparent when we consider what appears to be its wide distribution in nature. The question arises, however, as to whether "*Cl. putrificum*" should be regarded as identical with *B. putrificus* Bienstock. According to the arguments of Cunningham, it should not be so designated.

It is expedient at this time to review briefly the literature on *B. putrificus* Bienstock.³ Since Bienstock admitted that previous to 1906 he had been working with impure cultures, his earlier publications (1884, 1899, 1901) are of little value in establishing the identity of his microorganism. An incident occurred which was to play a very important part in the recognition (or lack of recognition) of his species of anaerobe. On December 14, 1899, the Kral Collection at Prague received a culture of "*B. putrificus*" from Bienstock. A comparison of dates reveals that Bienstock's culture was impure at that time. This situation makes it impossible to evaluate the studies in which the original culture of "*B. putrificus*" (Bienstock) has been employed, and partially accounts for the isolation of unrelated species of anaerobic bacteria from this culture. From subcultures of the original strain of "*B. putrificus*" Kleinschmidt (1925) and Zeissler (1928) obtained *B. amylobacter* and *B. putrificus-verrucosus*; Lehmann and Neuman (1927) identified *B. saccharobutyricus* and *B. putrificus-verrucosus*, and Cunningham (1931) isolated *B. sporogenes* from what must have been the same stock culture.

³ A more complete historical résumé of *B. putrificus* (Bienstock), *Cl. putrificum* (Reddish and Rettger) and *B. cochlearius* (McIntosh and Fildes) may be found in the senior author's doctorate dissertation deposited in the Yale University Library.

However, *B. putrificus* Bienstock came to be recognized as a definite species. It was not until the report of Robertson (1915-1916) appeared that any question arose regarding the purity of cultures which had been accepted as *B. putrificus* Bienstock. She held that a mixture of *Cl. tertium* and the malignant edema bacillus would present an appearance very similar to that described for *B. putrificus* cultures, particularly in so far as the morphology is concerned. Henry (1916-1917), McIntosh and Fildes (1917), the British Medical Research Committee (1919), Kendall, Day and Walker (1922) and recently Topley and Wilson (1929) have also expressed some doubt as to the existence of *B. putrificus* (Bienstock).

Reddish and Rettger (1921, 1922, 1923) compared the anaerobe isolated by Sturges and Rettger (1919) with the inadequate published descriptions of *B. putrificus* Bienstock, and concluded that the two organisms were identical and that they should be designated as *Cl. putrificum* Bienstock. The generic term, *Clostridium*, was applied merely to be consistent with current anaerobic nomenclature. This comprehensive study, as well as the subsequent work of Reddish (1923, 1924), served to establish "*Cl. putrificum*" as a definite species. As has been indicated, Cunningham (1932) does not doubt the individuality of the organism studied by Rettger and his associates, but does not believe that it should be regarded as a type strain of *B. putrificus* Bienstock, or as being in any sense identical with the alleged Bienstock anaerobe.

Since trustworthy strains of Bienstock's organism are not available, it is necessary to base one's conclusions on the published reports of the characteristics of this anaerobe, when making comparisons with other species of bacteria. The only report of the cultural reactions of *B. putrificus* Bienstock which can be used for this purpose is the 1906 account. It is significant to note that in this publication certain physiological properties were described in detail, whereas some important morphological and cultural characteristics were omitted. While the power of *B. putrificus* Bienstock to attack albumin, with the production of putrefactive by-products, was discussed at some length, the

decomposition of coagulated serum, gelatin, and other complex nitrogenous media, as well as the colonial features of this supposedly purified strain, were omitted.

Of the morphological features which have been described for *B. putrificus*, the types of sporulation are of considerable importance. That Bienstock observed two forms of spores in his culture is apparent from the following:

Au bout de peu de temps on ne voit plus que des baguettes de tambour et seulement cette forme de sporulation, tandis que dans l'albumine cuite le *Putrificus* montre assez souvent à côté des batonnets en baguettes de tambour une sporulation en forme de clostridium.

These observations cast considerable doubt on the purity of his culture. The reaction of his culture in milk also suggests the presence of an associated form, perhaps *Cl. sporogenes*. In this medium rapid growth took place, and peptonization and an offensive odor were noted after twenty-four hours incubation.

Many of the properties which appear necessary for the adequate characterization and differentiation of anaerobic forms at the present time are missing in Bienstock's description of *B. putrificus*. His observations of this organism were presumably made according to the standards of his time; hence, the incomplete characterization does not offer a substantial basis on which to recognize this alleged species. In view of the very meagre and somewhat questionable description of this species in 1906, and the inability to study trustworthy strains of *B. putrificus* Bienstock, the authors feel justified in not recognizing this species as a definite entity.

Since Cunningham (1930, 1931, 1932) isolated and characterized an anaerobic organism which he regarded as having more in common with *B. putrificus* Bienstock than any other anaerobe (particularly "*Cl. putrificum*") heretofore described, the question arises as to whether his species should be recognized as the type strain of *B. putrificus*. Since the present authors believe that the 1906 description of *B. putrificus* is inadequate, they cannot recognize Cunningham's B4a strains as type cultures of this organism. From the reactions of the two strains of Cunningham's organism employed in the present investigation it is

apparent that his "*B. putrificus*" possesses certain properties in common with *Cl. cochlearum*; however, the two anaerobes can be differentiated by their action on litmus milk and gelatin. Cunningham's *B. putrificus* peptonizes litmus milk and liquefies gelatin, while *Cl. cochlearum* does not attack the casein and gelatin. A more detailed investigation, including serological methods is advisable, before the exact relationship of these two organisms can be established.

That Cunningham's *B. putrificus* differs from the "*Cl. putrificum*" of Rettger and his associates can be shown in egg-meat medium, coagulated serum medium, brain medium and egg-cube medium. "*Cl. putrificum*" slowly, but definitely, digests these media, while Cunningham's organism is without action on them. Further differentiation of these organisms can be accomplished by observing their type of spores; "*Cl. putrificum*" produces round spores under given cultural conditions, while Cunningham's *B. putrificus* forms oval spores.

Because of suggestions by various writers of a close similarity, and perhaps identity, of "*Cl. putrificum*" and *Cl. cochlearum* (McIntosh and Fildes), the present paper deals largely with a comparative study of these two organisms. The general investigation included a study also of Cunningham's *B. putrificus*, (1932) strains of which were supplied by Dr. Cunningham. The results are not made a part of this report, because this organism proved to be so distinctly different from "*Cl. putrificum*," and because no question of priority could be raised, since Cunningham's anaerobe was isolated so recently.

Other organisms which have been thought to be more or less closely related to "*Cl. putrificum*" were also given some attention, particularly *Cl. tertium*,⁴ but they were eliminated soon because of obvious differences between them and "*Cl. putrificum*." It was concluded that the main effort should be devoted to determining the relative position of "*Cl. putrificum*" and *Cl. cochlearum* in the classification scheme.

⁴ The term "*B. tertius*" is claimed by some to be correct, because of its tolerance for atmospheric oxygen.

METHOD OF ISOLATION

In isolating "*Cl. putrificum*" Sturges and Rettger (1919) made use of the principle of associated growth. They plated mixtures of known *Staph. aureus* cells and preliminary cultures of the materials assumed from microscopic examination to contain this organism, and after incubation at 37°C., located colonies in which only *Staph. aureus* and "*Cl. putrificum*" were found to be present, by the usual staining and microscopic technique. These colonies were transferred to egg-meat medium. When, after incubation, definite proteolysis was noted "*Cl. putrificum*" was usually present in spore form; subsequent heating of this mixture resulted in a pure culture of this anaerobe. Obviously, the success of this method depended upon the chance selection of a colony containing only these two symbionts.

Since subsequent study by Reddish and Rettger (1923) revealed the ability of this anaerobe to decompose native protein, it appeared to the present writers that if this property were exploited, with modern methods for creating anaerobic conditions, the isolation of "*Cl. putrificum*" might be readily accomplished. A method was devised which included, (1) preliminary enrichment of the test material in egg-meat medium, (2) the use of the spore test, (3) the cultivation and selection of characteristic colonies, (4) the transfer of such colonies to cysteine meat infusion broth, then to egg-meat medium, and finally (5) the identification of the purified strain.

Material suspected of containing "*Cl. putrificum*" was seeded in egg-meat medium (Rettger, 1906) and incubated at 37°C. When stained preparations from this medium revealed large numbers of long, slender Gram-positive rods with round terminal spores, 2 cc. of the turbid supernatant liquid were transferred from the meat tube to 8 cc. of saline solution. These tubes were heated at 80°C. for twenty minutes, and the suspension streaked on freshly prepared cysteine meat infusion agar plates.

These plates were incubated at 37°C. in jars in which anaerobiosis was provided by alternate exhaustion with a vacuum pump and rinsing with pure carbon dioxide. This process was repeated

three times, and the jars sealed after the final exhaustion. It was apparent that, if surface colonies of "*Cl. putrificum*" were to be obtained consistently, rigid attention would have to be paid to the method of anaerobiosis.

After an incubation period of three days characteristic colonies (figs. 5 and 6) of "*Cl. putrificum*" were transferred to freshly boiled and cooled cysteine meat-infusion broth. The aid of a hand lens was necessary in order to see these colonies distinctly. The "ground-glass" appearance of the surface colonies was easily discernible under the lens. The broth tubes were placed in jars and anaerobic conditions provided according to the method described above. Incubation of the broth was at 37°C.

Because of the uniformly slow development of "*Cl. putrificum*" in all media, it was necessary to incubate the broth cultures from three to five days, depending upon the size of the colony used as inoculum. Two cubic centimeters of the broth were then transferred to sterile egg-meat medium and these tubes incubated until definite proteolysis had occurred. Gram stains prepared from these cultures at this time indicated the purity of the culture. Even when these preparations revealed only one type of a slender rod forming round, terminal spores, the culture was subjected at least three times to the same purifying process. When subtilis-group members or actively motile anaerobic forms were present it was necessary to resort to the deep-colony dilution method, using Veillon tubes and a basic cysteine meat infusion agar medium. As soon as these spreading forms were eliminated from the culture, the surface-inoculation method was used.

While certain investigators interested in anaerobic bacteria are not in agreement as to the best method of obtaining pure strains of a given anaerobic species, there is no doubt in the minds of the authors regarding the practicability of the above method for obtaining absolutely pure cultures of "*Cl. putrificum*." It should be emphasized, however, that rigid attention must be paid to the conditions of anaerobiosis and to critical examination of all cultures, particularly colony types, if trustworthy strains are desired. Morphological observations are indispensable, not only in following the isolation of this anaerobe, but also in the characterization of the pure form.

ORGANISMS OBTAINED FROM OTHER LABORATORIES

In addition to the authors' thirteen isolations, a number of strains were obtained from other investigators. Two strains of Sturges' original "*Cl. putrificum*" were received from him. Four strains (nos. 38, 220, 256 and 1333) regarded by Hall as *B. putrificus* were included in this work. The cultures of *Cl. cochlearum* included the type strain of the National Collection of Type Cultures (Hall's No. 3379) and seven other strains (nos. 166, 192, 446, 761, 3012, 3102a and 4070), which Hall had tentatively designated *B. cochlearius*. Two strains of Cunningham's *B. putrificus* were also included in this comparative study.

MORPHOLOGY AND STAINING CHARACTERISTICS

"*Cl. putrificum*" possesses a very characteristic morphology, especially when grown in egg-meat medium. Its cells are usually long, slender rods with rounded ends, the individual bacilli occasionally being slightly curved. The rods usually occur singly or in pairs; however, chains may be seen in old cultures. In non-sporing cultures there is often variation in the length of the rods, but the majority of the cells are from 0.4 to 0.6 μ broad and from 7.0 to 9.0 μ long. In young egg-meat cultures the rods may vary in length from 2.4 μ to 16.0 μ . These extreme types are the exception rather than the rule. Occasional pleomorphic changes, particularly a tendency toward filament formation, and toward a peculiar bulging appearance, have been observed with certain strains of this organism.

"*Cl. putrificum*" possesses peritrichous flagella and is feebly motile. With the advent of sporulation motility is partly, but not completely, retarded.

The spore of this species is unusually distinctive, and offers very material assistance in its identification. The egg-meat medium of Rettger is particularly suitable for such studies. The spores are produced in abundance and are the most characteristic of all of the types studied under these cultural conditions (fig. 1). They make their appearance in the medium at the time macroscopic disintegration of the meat is first noted, usually from seven to

ten days after inoculation. The spore is round, strictly terminal, and is very large as compared to the thickness of the rod. Spores are not formed readily in all media. They were not observed in broth, with or without glucose. Serum and blood media showed a few sporulating cells, but only after a long period of incubation.

In the present investigation cultures of *Cl. cochlearum* seemed to present their most characteristic morphology when grown in glucose infusion agar. Stained mounts from stab cultures in this medium revealed a straight, rather slender rod with rounded ends. Spores were formed in egg-meat medium, but were never observed in large numbers under these conditions. Sporing bacilli were occasionally seen in glucose infusion agar. The vegetative cells exhibited a tendency to vary in length (fig. 2) in this medium. The average length of the individual rods was observed to be $4.7\ \mu$; the thickness measured from 0.6 to $0.8\ \mu$. The bacilli usually occur singly or in pairs, rarely in chains. Curved forms, such as those seen in egg-meat cultures of "*Cl. putrificum*," are seldom produced.

Cl. cochlearum possesses peritrichous flagella and exhibits approximately the same degree of feeble motility as "*Cl. putrificum*."

The spores of *Cl. cochlearum*, as observed in plain meat infusion stab cultures, are oval; they are terminally situated and laterally distend the cell to approximately twice its diameter.

Both *Cl. cochlearum* and "*Cl. putrificum*" cultures stain well with ordinary aniline dyes; they are weakly Gram-positive in young, and almost uniformly Gram-negative in old cultures. Some cultures of *Cl. cochlearum* may show individual bacilli which appear to be more Gram-positive than other cells in the same culture; however, this difference was regarded as purely qualitative, and not one of differential value.

COLONY FORM

Subsurface colonies of "*Cl. putrificum*" and *Cl. cochlearum* are shown in figures 3 and 4. The colonies of "*Cl. putrificum*" occur as very delicate growths resembling tufts of cotton. In the early stages long, slender fibrils are seen radiating from a central

"nucleus." Later the centers become more dense. While the subsurface colonies of *Cl. cochlearum* are somewhat similar to the above in this early development, the radiating fibrils are more or less intertwined in the older colonies, forming peninsular or cochlear projections, as the name would suggest.

The surface colonies of "*Cl. putrificum*" on cysteine meat infusion agar (pH 7.2 to 7.4) are quite distinctive. By light transmitted at an angle through the colony, and under a strong hand lens, a definite "ground-glass" appearance (fig. 5) is noted. This feature facilitated the isolation and identification of "*Cl. putrificum*," and assisted materially in the differentiation of this anaerobe from *Cl. cochlearum*, although one strain of the latter organism possessed this property to a considerable degree. When surface colonies of "*Cl. putrificum*" are viewed under the low-power objective a distinctly furrowed appearance (fig. 6) is noted. The margin of the colony is more or less irregular and may show projections extending out onto the medium. The number of these projections and the density of the colony were found to vary with the moisture content of the medium at the time of inoculation. A very moist surface favored the development of a thin spreading growth having many offshoots, which is not unlike the colony of some "R" variants noted in certain members of the subtilis group. On a relatively dry surface the colonies are considerably more compact and of greater density.

The more compact colonies appear finely granular and amorphous under the microscope, and somewhat tenacious, though not mucoid, when touched with the needle. Macroscopically, the center of the colony is seen as relatively dense. Frequently the outer portion shows no real definition and appears to fuse with the medium. At the end of forty-eight hours incubation at 37° the colonies appear as small (about 0.5 mm. in diameter) round, entire, dew drop-like growths. They do not assume the distinctive "ground-glass" appearance until further aging.

The surface colonies of *Cl. cochlearum* (fig. 7) on cysteine meat infusion agar are appreciably larger than those of "*Cl. putrificum*." They are almost round and possess a slightly crenated margin. Concentric rings often appear at the periphery; however, these

may not be present if a thin spreading growth is produced, or if the colony is examined after an incubation period of less than three days. The interior of the colony is finely granular and amorphous, and often shows a slightly furrowed appearance which resembles to a certain extent that of "*Cl. putrificum*." While the surface colonies of *Cl. cochlearum* may show the "ground-glass" appearance, which so strongly features "*Cl. putrificum*" colonies, the former can be differentiated from the latter by its concentric rings. The accompanying photomicrograph was prepared from Hall's subculture of the original strain of *Cl. cochlearum*. Throughout this work Hall's culture produced very few "ground glass" colonies. However, a subculture of the original strain of *Cl. cochlearum* (no. 535) received directly from the British Type Culture Collection late in the course of this investigation developed surface colonies which were predominantly of the furrowed, "ground-glass" type. The marginal rings were always present, however, which permitted the ready differentiation of this colony from the surface colony of "*Cl. putrificum*."

While these species grew well on the surface of blood agar and serum media, their colony differences could not be observed through the colored plates in sufficient detail to warrant their being reported in the present comparative study. Neither species hemolyzed blood.

The authors are well aware of the tendency of bacteria generally to vary in their cellular and colonial morphology, but feel satisfied that under conditions which are definitely controlled as to temperature and cultural environment, cell and colony form are, as a rule, important characteristics in the classification of obligate anaerobes.

CULTURAL CHARACTERISTICS

Protein media. Since Reddish and Rettger (1923) have described the action of "*Cl. putrificum*" in egg-meat medium, it does not appear necessary to present a complete repetition here. This organism attacks the egg-meat mixture slowly, and in the course of time definitely reduces the volume of the solid material of this medium. It exhibits the characteristics of real putrefac-

tion. On the other hand, none of the various strains of *Cl. cochlearum* employed in the present investigation possessed proteolytic properties. The failure of this anaerobe to attack protein was demonstrated further by the use of brain and serum media. It differed also from "*Cl. putrificum*" in not liquefying casein and gelatin. While *Cl. cochlearum* grows well on these media it does not destroy complex nitrogenous substances in such a way that changes can be detected macroscopically. "*Cl. putrificum*" liquefies gelatin, peptonizes litmus milk, and digests brain, coagulated blood serum, egg and meat protein, with the evolution of the characteristic odors of putrefaction.

Broth. The growth of these microorganisms in cysteine meat infusion broth is not particularly abundant; however, the addition of a cube of coagulated egg-white to this medium results in an intense clouding throughout the medium. A dense layer of flaky precipitate develops around the egg cube, which increases in amount as incubation continues. *Cl. cochlearum* does not digest the egg-cube, while "*Cl. putrificum*" first attacks the edges of this material, then renders the entire cube transparent as proteolysis continues. After three weeks of incubation most of the cube dissolves; it often shows definite blackening, depending upon the strain employed. Agitation of the residue with a platinum needle reveals a more or less mucoid character.

BIOCHEMICAL REACTIONS

Fermentation tests. The basic medium employed in the fermentation studies was nutrient infusion broth to which had been added from 0.1 to 0.2 per cent cysteine hydrochloride. Valley (1929) has shown that this medium is quite satisfactory for fermentation studies on anaerobic species of bacteria. The following test substances were used: glucose, lactose, sucrose, maltose, mannitol, xylose, levulose and dextrin.

Cl. cochlearum failed to produce acid from these carbohydrates and mannitol; however, some gas was noted, which was in all probability of non-carbohydrate origin. Glucose was attacked only slightly by "*Cl. putrificum*," the medium never becoming distinctly acid. None of the other carbohydrates were attacked by this anaerobe.

Indol production. Freshly prepared Difco tryptophane broth was inoculated with a well developed egg-meat culture of the test organisms and the tubes incubated in anaerobic jars at 37°C. After three weeks incubation at this temperature all cultures were removed and the presence or absence of indol determined by means of the Bohme-Ehrlich test. Neither anaerobe gave any evidence of indol formation.

Hydrogen sulphide. The lead acetate medium usually employed in the determination of hydrogen sulphide production by members of the typhoid-paratyphoid-dysentery group was found to inhibit the growth of *Cl. cochlearum* and "*Cl. putrificum*." Though Hall (1924) advocated the use of brain medium to detect the ability of anaerobic bacteria to produce hydrogen sulphide, he explained that in the case of "*Cl. putrificum*" insufficient proteolysis usually takes place in this medium to liberate the iron present in the tissue and permit the formation of iron sulphide. His observations were corroborated in the present investigation; however, *Cl. cochlearum* and "*Cl. putrificum*" were found to produce considerable hydrogen sulphide when grown in egg-cube gelatin, in egg-cube broth, and in egg-meat medium. Filter paper which had been soaked in a 10 per cent solution of lead acetate and then dried was suspended immediately beneath the cotton stopper of tubes of the above media, after inoculation with the test species. In some instances where egg-cubes were employed, either in broth or in gelatin media, it was not necessary to use this paper, the presence of hydrogen sulphide being indicated by the blackening of the cubes.

Tyrosine. Old cultures of egg-meat medium of "*Cl. putrificum*" are usually dark brown, almost black, in appearance and, depending upon the strain, may or may not show crystals of tyrosine. This result is at variance with that of Hall (1922), who did not find his strains of "*Cl. putrificum*" capable of producing these crystals. The conditions which govern the production of this substance were not studied in detail; however, it can be said that these crystals have been observed only in old (one month or more) egg-meat cultures. *Cl. cochlearum* was not observed to produce tyrosine crystals.

Pathogenicity. Intraperitoneal injections of 1.5 cc. of 5-day old cysteine meat infusion broth cultures of the test species had no visible effect upon white mice.

CLASSIFICATION OF THE STRAINS STUDIED

In an earlier section of this paper it was stated that certain cultures which were received from Hall were tentatively designated *B. cochlearius* by him. In the course of this study it became apparent that some of these strains were, according to the results of the present investigation, not *Cl. cochlearum*, but "*Cl. putrificum*." In this group are found strains 166, 446, 3012, 3102A and 4070. Strains 38, 220, 256 and 1333 gave reactions which warrant their being designated "*Cl. putrificum*," while strain 761 could not be regarded as either *Cl. cochlearum* or "*Cl. putrificum*."

DISCUSSION AND SUMMARY

In the present investigation the organism of Reddish and Rettger, so-called "*Cl. putrificum*," was found to be entirely distinct from *Cl. cochlearum* and to have characteristics which set it apart from all other organisms. "*Cl. putrificum*" liquefies gelatin, peptonizes milk and slowly decomposes egg-meat, egg-cubes and coagulated serum. *Cl. cochlearum* was observed to be without these properties. The former organism produces round spores, under given conditions of cultivation, while those of the latter are oval.

That so-called "*Cl. putrificum*" is different from the organism which Cunningham studied and regards as *B. putrificus* Bienstock has been fully demonstrated by the writers. For a description and the results of comparative studies of "*Cl. putrificum*," *Cl. cochlearum* and Cunningham's organism the reader is referred to the senior author's doctorate thesis. Arguments have also been presented to show that the individuality of the strains which Bienstock described as *B. putrificus* is purely a matter of conjecture.

In his 1906 publication Bienstock described only three cultural reactions which would give any indication of the properties of his

organism. These reactions were (1) the form and position of the spores, (2) the decomposition of milk, and (3) the reaction on albumin. Since he observed two types of sporulation, there is a probability that his culture was still contaminated. Changes which he described as occurring in milk and in protein media may have been caused by a contaminant or by the combined action of the contaminant and the strain which he thought to be pure *B. putrificus*. There is too little concrete evidence in Bienstock's description to permit of a satisfactory comparison of his culture with any pure strain of an anaerobic species.

We may safely assume that the organism which Sturges and Rettger isolated and which Reddish and Rettger named "*Cl. putrificum*" is a distinct entity. It cannot be related definitely to what Bienstock described as *B. putrificus*, because of the lack of a satisfactory basis for comparison. That it is different from *Cl. cochlearum* and should never have been confused with it has been shown in the present investigation. The present authors agree with Cunningham (1932) that the organism which many regarded as similar to *B. putrificus* Bienstock is a definite and separate species; however, they cannot accept his suggestion and the assumption of others that it is intimately related to, or identical with, *Cl. cochlearum* (McIntosh and Fildes). It seems highly expedient, therefore, that "*Cl. putrificum*" should be re-named. The term "*Cl. lentoputrescens*" is suggested here as appropriate and meeting current taxonomic requirements. This name indicates one of the most outstanding properties of this organism, namely its slow (*L.-lentus*) decomposition (*L.-putresco*) of native proteins. The use of a new name for this species should dispel the confusion that exists today regarding the exact relationship of this species to other anaerobic bacteria.

It is to be remembered that the organism for which the name *Cl. lentoputrescens* is now proposed was apparently first observed by Rettger (1906, 1908) in his earlier studies on putrefaction, and isolated for the first time by Sturges and Rettger (1919).

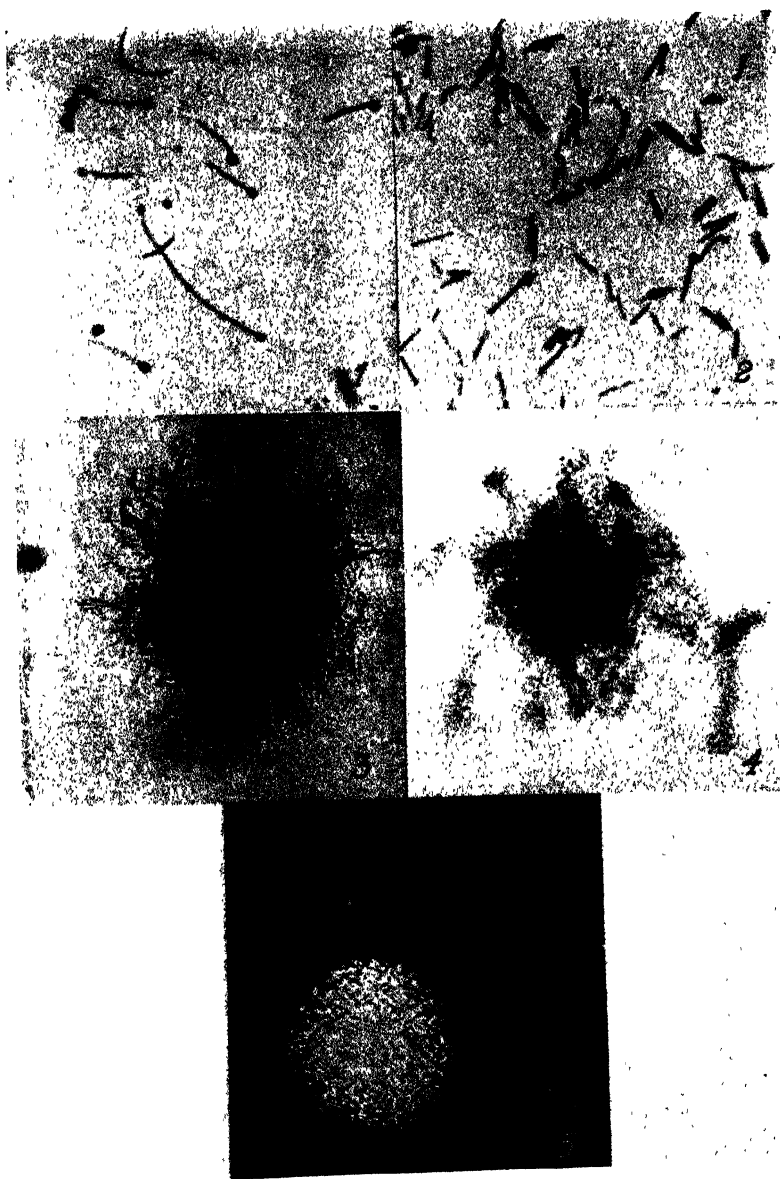
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PLATE 1

- Fig. 1. "*Cl. putrificum*" in egg-meat medium after 7 days incubation at 37°. × 1200.
- Fig. 2. *Cl. cochlearum* in egg-meat medium after 7 days incubation at 37°. × 1200.
- Fig. 3. "*Cl. putrificum*," sub-surface colony in meat infusion cysteine agar at 37°. × 125.
- Fig. 4. *Cl. cochlearum*, sub-surface colony in meat infusion cysteine agar at 37°. × 125.
- Fig. 5. "*Cl. putrificum*," surface colony on meat infusion cysteine agar, showing ground glass appearance. 72 hours, at 37°. × 15.

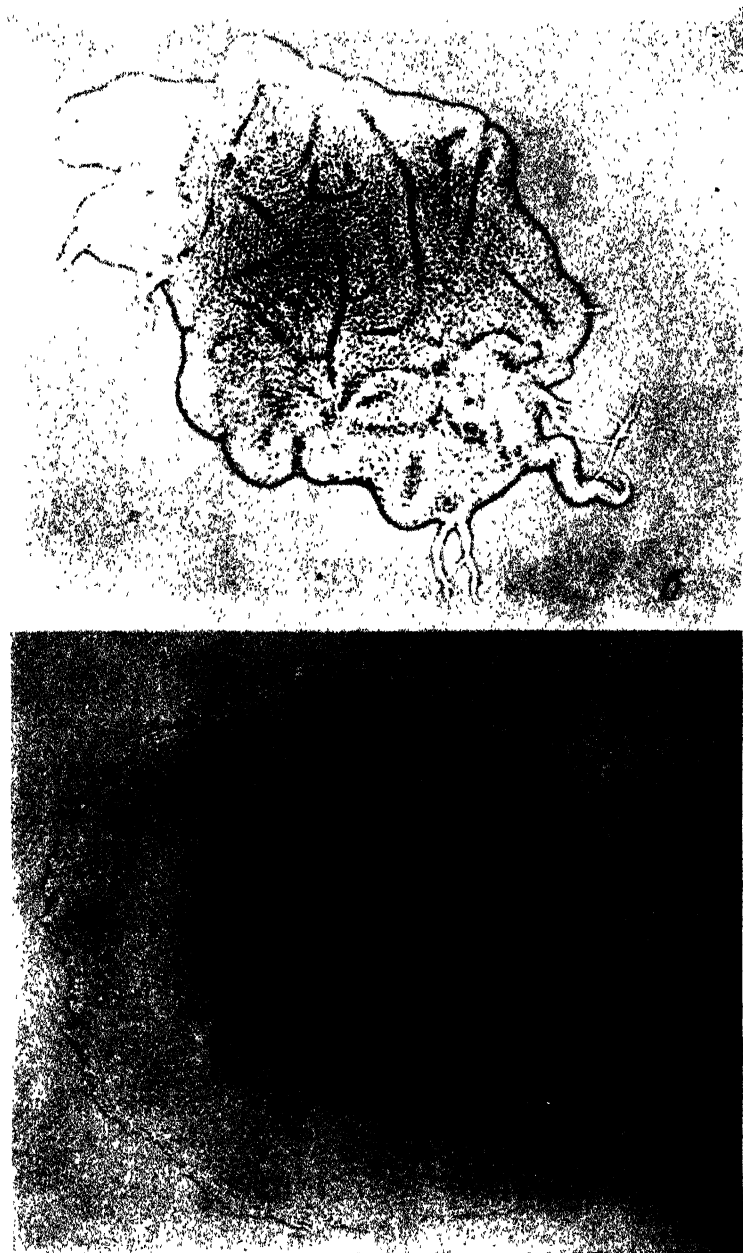


(Stanley E. Hartsell and Leo F. Rettger Taxonomic study of *clostridium putrificum*)

PLATE 2

FIG. 6. "*Cl. putrificum*," surface colony on meat infusion cysteine agar, showing furrowed appearance. 72 hours, at 37°. $\times 125$.

FIG 7. *Cl. cochlearum*, surface colony on meat infusion cysteine agar, showing irregular edge and concentric peripheral rings 72 hours, at 37°. $\times 50$.



(Stanley E. Hartsell and Leo F. Rettger. Taxonomic study of *Clostridium putrificum*.)

THE ANTISEPTIC EFFECT UPON TUBERCLE BACILLI OF CERTAIN RECENTLY-ADVOCATED COMPOUNDS

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Just as development of knowledge regarding disinfection has undergone decided changes during the past several decades so also have our views on the tubercle bacilli altered, especially in the direction of more workable and practical conceptions. The earlier workers had to be content to try, by very crude methods and using relatively poor nutrients for the tubercle bacilli, to discover the value of certain compounds as antiseptics. Our conceptions have undergone profound changes regarding the mode of action of such substances. Regarding the bacilli themselves the transition was from relatively crude and inaccurate quantitative tests to that of exact evaluation with fine graded suspensions and tests on media efficient for discerning few bacilli. This work has been particularly stressed by Corper and his colleagues who have been interested in chemotherapy and culture work (Corper, 1928; Corper and Uyei, 1929; Corper and Cohn, 1933).

Much time was spent by Wells, Corper, De Witt, and Lewis several decades ago in attempting to adapt the method of determining antiseptic coefficients for rapidly growing microorganisms to tubercle bacilli. The Rideal Walker method and its various modifications, and the Hygienic Laboratory method were tried; but like the more recently advocated Reddish modification of determining the phenol coefficient this comparison failed to give definite information for tubercle bacilli. One main objection to the tests adapted for rapidly growing microorganisms arose from the fact that liquid media were not well suited for growing tubercle bacilli, and the use of garnets or papers as transport ma-

terials proved too variable to yield results of practical value. Quantitative evaluation, so far as the tubercle bacilli were concerned, was not even thought of. Thus, all the work carried out with these methods left one in doubt as to the actual value of the data obtained. In addition the results did not take account of two other important factors; the effect upon small numbers of bacilli which in many cases could not be determined from the effect upon large numbers of bacilli; and the effect on the findings of a good nutrient as compared to a comparatively poor nutrient, as well as the effect of the presence of body fluids, tissues or protein solutions. At present there is no known liquid medium which will permit the growth of virulent human or bovine tubercle bacilli in plantings below about 1 mgm. (1 billion bacilli). Therefore, the criterion of death in this case would be concerned only with billions of bacilli.

Such single cell cultures as have been recorded for tubercle bacilli in liquid media have usually been concerned with rapidly-growing acid-fast bacilli, or with the more rapidly-growing avian tubercle bacilli. It has been the experience in our laboratory that macroscopic cultures of pathogenic human and bovine tubercle bacilli cannot be attained from small plantings of these microorganisms on the known liquid nutrient mediums. Much confusion has arisen from the use of acid-fast saprophytes as tubercle bacilli (Hastings and McCarter, 1932). Pathogenic tubercle bacilli of the human and bovine variety cannot be counted even with approximate accuracy by planting methods on any nutrients. Even were the Reddish (1926) test used, and planting consummated on a solid medium instead of a liquid medium the dilution factor aimed at in this test would be lacking. In certain cases the phenol coefficient may assume importance commercially and can easily be determined by the method used in this investigation; but since the important information desired concerns mainly the concentration of antiseptic capable of destroying tubercle bacilli the following report will give these figures alone. The methods used in this study may be classed under three groups as follows: (1) tuberculocidal-static tests; (2) tuberculocidal-time tests; and (3) tuberculocidal-time in the

presence of body fluids (blood was used). The tuberculocidal-static test is thus named because in it is recognized the fact that dilution or washing can only attain a dilution of the chemical tested rather than a complete removal. The tuberculocidal-time test gives due consideration to the time factor, being the actual killing time, since the dilution finally attained did not exert an effect on the bacilli as proved by test one. The third type of test is self-explanatory since it adds only the factor of testing the reagent in the presence of blood instead of a simple salt solution.

The technic of testing is therefore briefly as follows: for tuberculocidal-static test—various concentrations of the reagent to be tested in 1-cc. amounts are mixed with 1 cc. of a fine suspension of tubercle bacilli (Corper and Cohn, 1933) containing 1.0, 0.01 0.0001 or 0.000,001 mgm. of bacilli. This mixture is incubated for thirty minutes and is then diluted to 10 cc. with sterile 0.9 per cent sodium chloride solution and an appropriate amount (about 0.5 cc.) is planted on a number of crystal-violet potato-cylinder tubes. This test gives the optimum concentration of the reagent that is active as well as the static effect. For tuberculocidal-time test—to 0.5 cc. of the reagent in a 50-cc. centrifuge tube is carefully added 0.5 cc. of the suspension of bacilli. (The concentration of the suspension of bacilli is such that, when diluted to 50 cc., the plantings of bacilli amount to 0.1, 0.0001 and 0.000,001 mgm. per cubic centimeter respectively.) The mixture of bacilli and reagent is incubated for various time intervals and is then diluted to 50 cc. with sterile saline solution to eliminate, as far as possible, the tuberculostatic action of the reagent. The diluted mixture is then planted in appropriate amount, about 0.5 cc., on the crystal-violet potato-cylinder medium (the final concentration of the reagent is 1/100 of the original concentration). For determination of the tuberculocidal time in the presence of blood—to 5 cc. of citrated dog's blood (or other animal) is added a sufficient suspension of bacilli (about one to two months old) to give final concentrations per cubic centimeters of 0.1, 0.0001, or 0.000,001 mgm. To this is added 5 cc. of the test reagent. The suspension is mixed well and incubated

for various intervals after which it is diluted with 40 cc. of a sterile 0.9 per cent sodium chloride solution. In order to avoid too great bulk, only 10 cc. of this mixture is treated with 15 cc. of 6 per cent sulphuric acid solution and incubated for thirty minutes with occasional shaking. The solution is then neutralized with about 12 cc. of 5.4 per cent sodium hydroxide solution using brom-thymol-blue as indicator and keeping slightly on the acid side (pH 6.8). After centrifugation, the sediment is washed several times with about 40 cc. of sterile saline solution and, finally, the entire sediment is planted on the crystal-violet potato medium and the inspissated egg-yolk medium. In conformity with the plan outlined above, a group of antiseptics from various sources were submitted to the tuberculocidal-static test and the results are recorded in table 1; only those suggesting antiseptic value were then further submitted to the tuberculocidal time and blood tuberculocidal time tests.

It is to be noted from the data recorded in table 1 that while some of the preparations tested reveal a high tuberculocidal static value [Merthiolate 0.1 per cent, with heavy seedings, "Solution S. T. 37" (0.1 per cent crystalline hexylresorcinol dissolved in 30 per cent glycerol water, Leonard and Feirer, 1927). Di-Hydranol 0.1 per cent, and methyl and ethyl mercuri-succinimido aurate 0.1 per cent], others such as Acriflavine, Mercurochrome, Sanocrysin, and phenol reveal no effect in the concentrations (up to 1 per cent) used. It is also to be noted that while some of these compounds produce no effect on large numbers of bacilli one of them (Metaphen 0.001 per cent) is detrimental to small numbers of bacilli (0.000,01 mgm.).

The tuberculocidal-time value may be considered more exact in evaluating antiseptic values than the tuberculocidal-static test so that the results recorded in table 2, on some of the same compounds deemed worthy of further test from the results obtained with the tuberculocidal-static test, are more pertinent. The concentrations used in the tuberculocidal-time test were those recommended by the manufacturer or those found best suited from deductions derived from the tuberculocidal-static tests recorded in table 1. Some of them were also chosen (phenol 5

TABLE 1
Tuberculocidal-static value of antiseptics using virulent human tubercle bacilli (Gluckson) as test organism

REAGENT	AMOUNT OF BACILLI IN FINE SUSPENSION PER CUBIC CENTIMETER USED FOR TESTING									
	10.0 mgm.					0.1 mgm				
	10.0	5.0	2.0	1.0	0.1	1'000 0	100 0	10 0	1 0	0.000,01 mgm.
Concentration in per cent										
	10.0	5.0	2.0	1.0	0.1	1'000 0	100 0	10 0	1 0	0
"Merthiolate" (Sodium ethyl-mercurithio-salicylate).....	0*	0	0	0	0	+	+	+	+	+
Hexylresorcinol in 30 per cent glycerol (note).....	0	+	+	+	+	+	+	+	+	+
"Di-Hydranol" (Heptylresorcinol).....	0	+	+	+	+	+	+	+	+	+
"Metaphen" (4-Nitro-anhydro-hydroxy-mercuri-ortho-cresol).....	+	+	+	+	+	+	+	+	+	+
Acridlavine (Diamino-methylacridine chloride neutral).....	+	+	+	+	+	+	+	+	+	+
"Mercurochrome 220-Soluble" (Dibrom-oxymercuri-fluorescein).....	+	+	+	+	+	+	+	+	+	+
"Sanocrysin" (A gold sodium thio-sulphate).....	±†	±	±	±	±	+	+	+	+	+
Ammonium-succinimido aurate.....	+	+	+	+	+	+	+	+	+	+
Methyl-mercuri-succinimido aurate	0	0	0	0	0	+	+	+	+	+
Ethyl-mercuri-succinimido aurate	0	0	0	0	0	+	+	+	+	+
"Neo-Vonargen" (Diamindisulphoricincolargantium).....	+	+	+	+	+	+	+	+	+	+
Phenol.....	+	+	+	+	+	+	+	+	+	+

*The findings are recorded as 0 = no growth obtained on the culture tubes (3 for each test); ± = growth was retarded and only occurred on one or two of the three tubes, and + = a growth equal to that of the control tubes.

† In 2 per cent concentration mercurochrome killed the tubercle bacilli both in 10 and 0.1 mgm. tests.

‡ The ammonium succinimido aurate, methyl, mercuri-succinimido aurate and ethyl mercuri succinimido aurate were prepared and kindly supplied for this study by Mr. Ben Sher, Chief Chemist of the City of Chicago Municipal Tuberculosis Sanitarium.

§ Phenol in 3 per cent concentration was able to prevent growth of the bacilli and all the culture tubes were negative for this concentration.

Note: All solutions of Hexylresorcinol were prepared from the pure crystalline chemical kindly supplied for this purpose by Sharp and Dohme. A 0.1 per cent solution was prepared with 30 per cent glycerol and is known under the trade name of "Hexylresorcinol Solution S.T. 37." Higher dilutions were made with physiologic saline solution. "Solution S.T. 37" is also used in the literature to designate the Liquor Hexylresorcinolis 1:1000.

TABLE 2
Tuberculocidal-time value of antiseptics using virulent human tubercle bacilli (Gluckson) as test organism

REAGENT AND CONCENTRATION USED	AMOUNT OF BACILLI IN FINE SUSPENSION PER CUBIC CENTIMETER USED FOR TESTING																							
	10.0 mgm.								0.1 mgm.								0.0001 mgm.							
	Time of exposure in minutes																							
	Immediately						Control						Immediately						Control					
	2	5	10	15	20	30	45	60	2	5	10	15	20	30	45	60	2	5	10	15	20	30	45	60
"Merthiolate" solution 45-1:1000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
"Solution S.T. 37" 1:1000†	+	+	+	±	0	0	0	0	+	0	0	0	0	0	0	0	+	0	±	0	0	0	0	0
"Di-Hydranol" 1:1000	+	+	0	0	0	0	0	0	+	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0
"Di-Hydranol" in 20 per cent ethyl alcohol 1:1000	+	+	0	0	0	0	0	0	+	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0
"Metaphen" 1:1000	+	+	0	0	0	0	0	0	+	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0
"Acridlavine" 1:1000	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	+	+	0	0	0	0	0	0
"Merurochrome" 1:50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl-mercuri-succinimido aurate 1:1000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ethyl-mercuri-succinimido aurate 1:1000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	0	+	+	+	+	0	0	0	0
Ethyl alcohol 95 per cent.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	+	+	+	+	±	0	0	0
Ethyl alcohol 20 per cent.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenol 1:20	±	0	0	0	0	0	0	0	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* The culture findings are recorded in this table in the same manner as given for table 1. (See footnotes of table 1.)

† "Solution S.T. 37" is the trade name of a 0.1 per cent solution of hexylresorcinol in 30 per cent glycerol. The dilutions (1:1000 etc.) given in the table designate the concentration of active chemical ingredient.

TABLE 3
Tuberculocidal-time value of antiseptics in the presence of blood,* using virulent human tubercle bacilli (Gluckson) as test organism

REAGENT AND CONCENTRATION USED	AMOUNT OF BACILLI IN FINE SUSPENSION PER CUBIC CENTIMETER USED FOR TESTING																				
	0.1 mgm.										0.0001 mgm.										0.000,001 mgm.
	Time of exposure																				
	Immediately	2 minutes	5 minutes	10 minutes	15 minutes	30 minutes	1 hour	2 hours	6 hours	24 hours	Immediately	2 minutes	5 minutes	10 minutes	15 minutes	30 minutes	1 hour	2 hours	6 hours	24 hours	
"Merthiolate" 1:1000.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
"Solution S.T. 37" 1:1000†.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
"Di-Hydranol" 1:1000.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
"Metaphen" 1:1000.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

* The blood used in these tests was a citrated dog blood prepared by running nine volumes of sterile dog blood into one volume of 3 per cent neutral trisodium citrate solution.

† The findings are recorded as 0 = no growth, ± = a retarded growth in only one or two of six culture tubes and + = a growth equivalent to the control in all the culture tubes. A dash "-" signifies that no test was made at the interval designated.

‡ The amount of active ingredient is designated by the proportion 1:1000 (0.1 per cent).

per cent and alcohol 95 per cent) because the concentrations are those commonly recommended for practical use.

The results of the tuberculocidal-time values are recorded in table 2, and indicate, somewhat surprisingly, that Merthiolate proved of no tuberculocidal value within sixty minutes for large numbers of bacilli, although the static value may be fairly high as indicated in table 1. For small numbers of bacilli a tuberculocidal action is exerted after about fifteen to twenty minutes. The same conclusion applied to the methyl or ethyl mercuri-succinimido aurate; "Solution S. T. 37" and Di-Hydranol, however, showed in contrast a high tuberculocidal efficiency, killing even heavy concentrations of bacilli within about ten minutes and lower concentrations of bacilli within two minutes.

Mercurochrome (2 per cent) and Acriflavine (0.1 per cent) and ethyl alcohol (20 per cent) proved innocuous within one hour's time of exposure. Metaphen (a mercury compound) in tuberculocidal tests killed in thirty minutes heavy concentrations of bacilli while few bacilli were killed within five minutes. Ethyl alcohol 95 per cent, killed all concentrations of bacilli within five to ten minutes while phenol (5 per cent) killed almost on contact.

In the presence of blood, chosen as a general protein material, the tuberculocidal values for some of these preparations change markedly. "Solution S. T. 37" and Di-Hydranol proved highly inefficient in the strength available for practical use. On the other hand Merthiolate (0.1 per cent) and Metaphen (0.1 per cent) proved tuberculocidal for either large or small numbers of bacilli after about one hour of contact in blood.

DISCUSSION

Although tubercle bacilli have been tested innumerable times both in antiseptic and chemotherapeutic experiments, particularly by groups of workers in different countries several decades ago, the work has waned particularly during the past decade because of the fruitlessness of such studies especially with regard to chemotherapy but also with regard to the action of antiseptics. The explanation of these failures lies mainly in the crude pro-

cedures used and especially in the lack of satisfactory culture methods or more specifically the lack of their accurate evaluation. Although references are made in a number of places within the past decade to antiseptic tests with tubercle bacilli included with other microorganisms, upon examination no such tests are found included in the studies or non-pathogenic rapid-growing acid-fast bacilli alone were used. There are, however, two studies of chemical preparations which are included among the substances tabulated in this study which merit consideration. One of these was on Metaphen by M. Jacobs in 1932, who found that a solution of Metaphen in oil (1:3000) was bactericidal (?) after five hours contact although no effort was made to remove the reagent and a relatively poor nutrient medium was used while, in addition only a heavy suspension was tested. The other concerned Sanocrysin (Sweany and Wasick, 1925) and this reagent was found to inhibit the growth of tubercle bacilli in very high dilution (about 1:1,000,000) but did not show any tuberculocidal action in concentrations as low as 1:2000. The same criticisms of the tests offered above applies to this study, e.g., poor nutrients and only heavy plantings being used. It is obvious that such studies can give practically no insight as to antiseptic values.

If compounds are to be studied the information must include, particularly, the effect on small numbers of tubercle bacilli as well as on larger ones; they must be concerned with tubercle bacilli (variety specified) and not with acid-fast rapid-growing saprophytes alone; they must give information regarding tuberculocidal as well as tuberculostatic effect, and they must include a complex protein mixture such as blood or some other equally serviceable material which cannot be replaced by using an agar cup (Allen and Wright, 1931).

The methods used in the study reported here includes a tuberculostatic study using graded amounts of bacilli and media qualified to grow small numbers, a tuberculocidal study aimed to correlate the foregoing and to remove by dilution the antiseptic reagent as far as is practically possible, and a good nutrient capable of supporting growth of a few bacilli as a test, and finally a tuberculocidal-time test in the presence of body proteins aimed

at excluding the possibility of an interference with antiseptic action by body protein materials.

SUMMARY

1. The usual methods of testing antiseptics cannot be directly adapted to use for tubercle bacilli.

2. A group of three procedures is described for testing antiseptics, utilizing particularly a wide range of graded planting and nutrient media qualified for growing small numbers of tubercle bacilli. The procedures consist of a tuberculocidal-static test, a tuberculocidal-time test and a tuberculocidal-time test in the presence of body proteins (blood).

3. A number of recently recommended antiseptics including Merthiolate, "Solution S. T. 37" Di-Hydranol, Metaphen, Mercurochrome, Sanocrysin, and Acriflavine and a few standard materials such as phenol and ethyl alcohol are tested.

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THE DIFFERENTIATION OF HEMOLYTIC STREPTOCOCCI OF HUMAN AND ANIMAL ORIGIN BY GROUP PRECIPITIN TESTS¹

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In previous studies (Edwards 1932, 1933, 1933a) on the low-acid-producing, non-sodium-hippurate-hydrolyzing, hemolytic streptococci of animal origin, it was demonstrated that these organisms could usually be differentiated from streptococci of human origin by their fermentation reactions and their action on methylene blue. The methods used in differentiating these organisms can be presented best in tabular form. The results obtained in a study of 175 cultures of animal origin and 120 cultures of human origin are given in table 1.

It will be noted that *Str. equi* was differentiated from all the other cultures studied by its failure to ferment either lactose, sorbitol, or trehalose. This organism was found only in strangles, or distemper, of the horse. Of the remaining animal cultures, 95.8 per cent were classified as type A. They were distinguished from other cultures by the fact that they fermented sorbitol and did not attack trehalose. This type is widely distributed among the domestic and laboratory animals. The remaining animal cultures were classified as type B. Cultures of this group resemble human cultures in their action on sorbitol and trehalose. However, all the strains of this group reduced methylene blue, while only 5 per cent of the human cultures reduced the dye.

These results have been partially confirmed by Lancefield

¹ The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

(1933), who worked with *Str. equi* and representatives of type A as well as with high-acid-producing bovine cultures and cultures of human origin. Lancefield found that *Str. equi* and the type A animal streptococci could be differentiated from human streptococci by group precipitin tests, as well as by fermentative reactions. No representatives of type B streptococci were included in her work.

While fermentative characters offer a practical method for the differentiation of *Str. equi* and the type A animal strains from the streptococci of human origin, the relation of the type B animal strains to human streptococci may be questioned. The reduction

TABLE 1
Biochemical reactions

SOURCE	NUMBER OF CULTURES	LACTOSE	SORBITOL	TRHALOSE	REDUCTION OF METHYLENE BLUE
Animal:					
<i>Str. equi</i>	9	—	—	—	—
Type A.....	159	+	+	—	—
Type B.....	7	±	—	+	+
Human.....	120	±	—	+	114— 6+

of methylene blue is a quantitative test and a few human cultures reduce the dye quite as actively as the type B animal strains. The purpose of the present paper is to confirm Lancefield's observations on the precipitin reactions of the type A streptococci and to present further evidence that the type B strains are distinct from the human streptococci.

METHODS

The methods used in studying fermentation and the reduction of methylene blue are those reported by Edwards (1933). In performing the group precipitin tests the method of Lancefield (1933) was used. In the preparation of antigens the organisms were centrifuged from 250 cc. of an eighteen-hour broth culture and sus-

pended in 5 cc. of physiological saline. Normal hydrochloric acid was added until the suspension was sufficiently acid to turn Congo-red paper blue. The acidified suspension was placed in a boiling water bath for ten minutes, removed and cooled rapidly. The organisms were centrifuged out and the supernatant fluid poured off and made neutral to litmus with sodium hydroxide. After neutralization a pronounced precipitate was formed. The liquid was cleared by centrifugation, and the water-clear supernatant fluid employed as antigen in the tests.

In the preparation of antisera, formalin-killed cultures were injected daily for even days. This was followed by a seven-day rest period, after which the daily injections were repeated. Satisfactory sera were obtained after two to four series of injections.

In performing the test, 0.2 cc. of undiluted antiserum was used in each tube. Antigen was used in amounts of 0.4 cc., 0.1 cc. and 0.025 cc. The total volume of the test was 0.6 cc. Adequate serum and antigen controls were provided in all cases. The antigens were layered over the serums and the tests examined for ring formation after thirty minutes at room temperature. The tubes were then shaken, placed in the incubator at 37° for two hours and then read. A final reading was taken after storage in the ice box overnight.

Positive tests gave marked ring formation and a heavy precipitate on incubation, while negative tests remained perfectly clear. No weak or doubtful tests were encountered, the results being clear cut in all instances.

RESULTS

The results of the precipitin tests, together with the fermentation reactions and the action of the cultures on methylene blue, are recorded in table 2. Three cultures of *Str. equi*, 29 strains of type A animal streptococci, 7 strains of type B animal streptococci, and 26 strains of human streptococci are included in the table. It will be noted that *Str. equi* and types A and B of the animal streptococci all cross precipitate. No differences were discernible in homologous and heterologous reactions with the sera and antigens employed in this study. None of 38 animal strains gave any reac-

TABLE 2

ORIGIN OF CULTURES			FERMENTATION REACTIONS			REDUCTION OF METHYLENE BLUE	PRECIPITIN TESTS			
Strain	Source	Disease	Lactose	Sorbitol	Trehalose		Antisera for			
							Str. equi (D1)	Animal type A (2)	Animal type B (36)	Human (823)
Streptococcus equi										
D1	Horse	Strangles	-	-	-	-	+	+	+	-
D2	Horse	Strangles	-	-	-	-	+	+	+	-
D3	Horse	Strangles	-	-	-	-	+	+	+	-
Type A animal streptococci										
1	Horse	Chronic endometritis	+	+	-	-	+	+	+	-
2	Horse	Chronic endometritis	+	+	-	-	+	+	+	-
8	Horse	Chronic endometritis	+	+	-	-	+	+	+	-
9	Horse	Chronic endometritis	+	+	-	-	+	+	+	-
12	Horse	Chronic endometritis	+	+	-	-	+	+	+	-
18	Horse	Chronic endometritis	+	+	-	-	+	+	+	-
26	Horse	Chronic endometritis	+	+	-	-	+	+	+	-
33	Horse	Chronic endometritis	+	+	-	-	+	+	+	-
10116	Horse	Aborted fetus	+	+	-	-	+	+	+	-
10986	Hog	Septicemia	+	+	-	-	+	+	+	-
25273	Hog	Aborted fetus	+	+	-	-	+	+	+	-
611B	Guinea pig	Lymphadenitis	+	+	-	-	+	+	+	-
Gp1F	Guinea pig	Lymphadenitis	+	+	-	-	+	+	+	-
3962	Chicken	Slipped tendon	+	+	-	-	+	+	+	-
6554	Chicken	Slipped tendon	+	+	-	-	+	+	+	-
3580	Fox	Pneumonia	+	+	-	-	+	+	+	-
Clark	Rabbit	Septicemia	+	+	-	-	+	+	+	-
25261	Cow	Abortion	+	+	-	-	+	+	+	-
25505	Cow	Septicemia	+	+	-	-	+	+	+	-
28117	Cow	Septicemia	+	+	-	-	+	+	+	-
126A	Cow	Metritis	+	+	-	-	+	+	+	-
E1	Cow	Mastitis	+	+	-	-	+	+	+	-
E2	Cow	Mastitis	+	+	-	-	+	+	+	-
E5	Cow	Mastitis	+	+	-	-	+	+	+	-
E7	Cow	Mastitis	+	+	-	-	+	+	+	-
E8	Cow	Mastitis	+	+	-	-	+	+	+	-
E9	Cow	Mastitis	+	+	-	-	+	+	+	-
E12	Cow	Mastitis	+	+	-	-	+	+	+	-
E13	Cow	Mastitis	+	+	-	-	+	+	+	-

TABLE 2—*Concluded*

ORIGIN OF CULTURES			FERMENTATION REACTIONS			REDUCTION OF METHYLENE BLUE	PRECIPITIN TESTS			
Strain	Source	Disease	Lactose	Sorbitol	Trehalose		Antisera for			
							Str. equi (D1)	Animal type A (2)	Animal type B (36)	Human (S23)
Type B animal streptococci										
4	Horse	Slight cervicitis	-	-	+	+	+	+	+	-
5	Horse	Slight cervicitis	+	-	+	+	+	+	+	-
36	Horse	None	-	-	+	+	+	+	+	-
55	Horse	None	-	-	+	+	+	+	+	-
F6	Horse	Abortion	-	-	+	+	+	+	+	-
126B	Cow	Metritis	-	-	+	+	+	+	+	-
25272	Hog	Abortion	-	-	+	+	+	+	+	-
Human streptococci										
S23	Human	Pneumonia	+	-	+	-	-	-	-	+
S43	Human	Pneumonia	+	-	+	-	-	-	-	+
NY5	Human	Scarlet fever	+	-	+	-	-	-	-	+
C203	Human	Scarlet fever	+	-	+	-	-	-	-	+
H3	Human	Mastoiditis	+	-	+	-	-	-	-	+
H4	Human	Mastoiditis	+	-	+	-	-	-	-	+
H5	Human	Mastoiditis	+	-	+	-	-	-	-	+
H11	Human	Meningitis	-	-	+	-	-	-	-	+
H28	Human	Scarlet fever	+	-	+	-	-	-	-	+
H34	Human	Scarlet fever	+	-	+	-	-	-	-	+
H36	Human	Measles	+	-	+	-	-	-	-	+
H39	Human	Puerperal sepsis	+	-	+	-	-	-	-	+
H40	Human	Puerperal sepsis	+	-	+	-	-	-	-	+
H42	Human	Tonsillitis	+	-	+	-	-	-	-	+
H46	Human	Erysipelas	+	-	+	-	-	-	-	+
H51	Human	Erysipelas	+	-	+	-	-	-	-	+
H56	Human	Pleuritis	-	-	+	-	-	-	-	+
H57	Human	Erysipelas	+	-	+	-	-	-	-	+
E41	Human	Sore throat	+	-	+	-	-	-	-	+
E42	Human	Sore throat	+	-	+	-	-	-	-	+
E44	Human	Otitis	+	-	+	-	-	-	-	+
E46	Human	Sore throat	+	-	+	-	-	-	-	+
E50	Human	Erysipelas	+	-	+	-	-	-	-	+
E53	Human	Sore throat	+	-	+	-	-	-	-	+
E63	Human	Sore throat	+	-	+	-	-	-	-	+
E67	Human	Sore throat	+	-	+	-	-	-	-	+

tion with sera derived from human cultures. The 26 cultures of human streptococci all gave marked precipitates with sera derived from human strains and failed to react with sera derived from *Str. equi* and types A and B animal streptococci. In addition to the antiserum for the strain S23, another serum prepared from a human strain was tested with several of the antigens. This serum, kindly furnished by Dr. R. C. Lancefield, gave identical results with the serum derived from strain S23. In addition to the antisera included in table 2, sera were prepared from three other strains of the type B animal streptococci and two additional cultures of type A animal streptococci. Since results obtained with these sera were identical with those recorded, they have not been included in the table.

DISCUSSION

In the interpretation of the results reported here, two facts must be kept in mind. First, only the low-acid-producing, non-sodium hippurate-hydrolyzing streptococci are considered in this work. This is the group which Smith (1929), Diernhofer (1930), Seelemann and Hadenfeldt (1930, 1932), Hergesell (1931), and Minett and Stableforth (1931) were unable to distinguish from human streptococci. Second, no claim is made that the precipitin tests reported here are type-specific, or capable of detecting types present in a relatively broad group. In extensive studies of the antigenic complex of hemolytic streptococci, Lancefield (1925, 1928, 1933), found a number of different reactive substances in the group. In acid extracts of hemolytic streptococci this investigator found two antigens, a type-specific protein and a group-specific carbohydrate. The present work was done with antigens prepared from acid extracts of the organisms. Such crude extracts contain both specific and group antigens. The differentiation of the human and animal strains is based upon the group antigens contained in these extracts. Further work must be done before serological types which may be present within the group of animal streptococci can be distinguished.

The human and animal strains employed in this study can be distinguished easily by the precipitin test. The human cultures

all belong to one well-defined group. This group gives no cross reactions with any of the animal cultures. The animal cultures also form one group. Cross precipitation occurs between the types whose biochemical characters differ. It is extremely interesting that the type B animal strains, which closely resemble the human cultures in their biochemical characters, possess a group antigen which reacts similarly to the group antigen of *Str. equi* and the type A animal strains. This fact confirms the opinion expressed in a previous publication (Edwards, 1933), that the type B animal cultures differ from strains derived from pathological conditions in human beings.

Several of the animal strains (E1, E2, E5, E7, E8, E9, E12, E13) were received from another laboratory as cultures of *Str. epidemicus*. These cultures were isolated from the milk of cows affected with mastitis. Attention has been called (Edwards 1933a), to the fact that their biochemical characters were identical with those of the type A animal streptococci. Their serological relations also indicate that they are of animal origin. On the contrary, cultures derived from epidemics of septic sore throat (E41, E42, E44, E46, E50, E53, E63, E67), resemble other streptococci of human origin in their biochemical and serological properties.

SUMMARY

It was possible to differentiate hemolytic streptococci of human and animal origin by the precipitin test when acid extracts of the organism were used as antigens. *Str. equi*, type A animal streptococci, and type B animal streptococci all belong to the same serological group. Streptococci of human origin constitute a second group. No cross reactions occurred between the two groups.

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A NOTE ON THE HYDROLYSIS OF SODIUM HIPPURATE BY THE HEMOLYTIC STREPTOCOCCI¹

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Since Ayers and Rupp (1922) demonstrated that the high-acid-producing streptococci of bovine origin were able to hydrolyze sodium hippurate while hemolytic streptococci of human origin did not, the hydrolysis of this substance has been widely used as a test in the classification of streptococci. It has been stated by Smith (1929), Minett and Stableforth (1931), Edwards (1932, 1933) and Lancefield (1933) that the low-acid-producing, actively hemolytic streptococci of animal origin resemble streptococci of human origin in not being able to hydrolyze sodium hippurate. All these workers relied on the simple ferric chloride test of Ayers and Rupp for the detection of hydrolysis. In this test ferric chloride solution is placed directly in the culture medium and the formation of an insoluble precipitate is recorded as a positive test for the presence of benzoic acid.

On the contrary, Klimmer and Haupt (1926) and Klimmer, Haupt and Roots (1928) found that the low-acid-producing, sorbitol-fermenting streptococci of horses as well as the high-acid-producing bovine strains were able to split hippurate. The equine cultures, they stated, hydrolyzed only slight amounts of hippurate and the resulting benzoic acid was so slight in amount that it was detectable only by a special technique. These workers acidified 2 cc. of a hippurate broth culture of the streptococcus to be tested with sulfuric acid and extracted the culture with

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ether. The ether was evaporated and the residue taken up with petroleum ether. The petroleum ether extract was filtered, the petroleum ether evaporated and the residue sublimed. The sublimate was dissolved in dilute ammonia and the excess ammonia driven off by heating on a water bath. Ferric chloride solution was added and the formation of a flesh colored precipitate was considered indicative of the presence of benzoic acid and recorded as a positive test for the hydrolysis of sodium hippurate.

Klimmer and Haupt (1926) found that sorbitol-fermenting streptococci of horses constantly hydrolyzed slight amounts of hippurate and that the benzoic acid could be detected only in the petroleum ether residues. Klimmer, Haupt and Roots (1928) confirmed these observations and stated, in addition, that strains of human streptococci did not hydrolyze hippurate. If this work could be confirmed, using a large number of strains, the method would offer possibilities as a means of differentiation between hemolytic streptococci of human and animal origin. Accordingly, the writers have repeated the work of Klimmer and Haupt, using cultures from a variety of domestic animals, including horses, and from various pathologic processes in human beings.

With two exceptions the technique was the same as that employed by Klimmer and Haupt. In our tests 10 cc. of culture fluid were used instead of 2 cc. It was thought that if hydrolysis occurred the benzoic acid produced in the larger amount of culture would be much more easily demonstrable. Moreover, the petroleum ether residues were not sublimed. Sublimation was omitted from the method for two reasons. First, it was found that no precipitate was formed when ferric chloride was added to the ammoniacal petroleum ether residues of uninoculated hippurate broth. Second, there was never sufficient residue obtained from petroleum ether extracts to permit sublimation.

In all, 35 cultures of hemolytic streptococci from horses, cows, chickens, hogs and guinea pigs have been tested. This group was composed mainly of sorbitol-fermenting strains (type A of Edwards, 1933) but a few non-sorbitol-fermenting strains (type B of Edwards, 1933) were included in the group. The majority of these cultures were isolated from horses and correspond to the

Str. abortus-equi and *Str. pyogenes-equi* of Klimmer and Haupt. Nineteen cultures of human origin were used for comparison.

The claims of Klimmer and Haupt that the sorbitol-fermenting streptococci of horses regularly hydrolyze hippurate could not be confirmed. Among the 35 animal cultures tested, traces of benzoic acid were detectable in the petroleum-ether residues of only 5. Even when 10 cc. of culture were used in the tests only slight traces of benzoic acid were present. The amounts were so small that the tests could only be recorded as doubtful. On the contrary, a culture of *Str. agalactiae* used for comparison gave a pronounced test for benzoic acid when only 0.1 cc. of culture fluid was used for the test. It may be seen that the amounts of sodium hippurate hydrolyzed by low-acid-producing streptococci of animal origin are so slight as to be negligible. In most instances, hydrolysis is not detectable by the method employed.

As an additional check, 50 cc. of four-day-old cultures of strains which had presumably yielded traces of benzoic acid in the petroleum-ether residues were steam distilled. In all, 500 cc. of distillate were collected and aliquots titrated against potassium hydroxide. This quantitative method failed to detect benzoic acid as there was no increase in volatile acid above that found in broth to which hippurate had not been added.

Of the 19 cultures of human origin tested, 3 produced some hydrolysis. The amount of benzoic acid formed was approximately the same as that produced by the 5 animal strains that gave evidence of hippurate splitting.

SUMMARY

Thirty-five strains of low-acid-producing, hemolytic streptococci of animal origin and 19 strains of human origin were tested for their ability to hydrolyze sodium hippurate. The method of Klimmer and Haupt was used in the tests. Five of the animal strains and 3 of the human cultures gave evidence of having hydrolyzed very slight amounts of hippurate. The statement of Klimmer and Haupt that the sorbitol-fermenting streptococci of horses constantly hydrolyze sodium hippurate could not be confirmed.

ADDENDUM

Since the above paper was presented for publication the writers have received two cultures, labelled *Str. abortus equi* I and II, from Dr. Haupt of the University of Leipzig. We have confirmed the statement of Klimmer and Haupt (1926) that these cultures hydrolyze sodium hippurate. These cultures differ from the cultures we have isolated from horses in many respects. The German cultures do not belong to the group of low-acid-producing streptococci. In glucose broth they produce a reaction of pH 4.4. They reduce litmus and coagulate milk, while our cultures do not. The German cultures ferment sorbitol and trehalose, while none of the 350 cultures of low-acid-producing streptococci which we have studied ferments both of these substances. The cultures of Dr. Haupt seem to be more closely related to the high-acid-producing streptococci found in bovine mastitis than to the group with which we have worked.

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THE BACTERIOLOGY OF SWISS CHEESE

I. GROWTH AND ACTIVITY OF BACTERIA DURING MANUFACTURING PROCESSES IN THE SWISS CHEESE KETTLE

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The bacteriology of Swiss cheese may be divided into four parts: (1) bacteriology of the milk before processing, (2) bacteriology of milk, curd, and whey in the cheese kettle, (3) bacteriology of the cheese in the press, and (4) bacteriology of the cheese in the cellars. This paper will be confined to the bacteriology of the kettle contents from the time the milk enters the kettle until the curd is dipped.

The number, kind, state of activity, and amount of previous growth of bacteria of the kettle milk influence greatly the behavior of the milk and curd in the kettle and the character of the cheese in the press. The previous growth of bacteria in the milk will have produced a certain amount of "ripeness," a characteristic which influences physico-chemical and bacteriological changes in the curd in the kettle and press.

The manufacturing process in the kettle, from the time of addition of starters and rennet to the time of dipping the curd, requires from one and one-half to three hours. The length of time varies with a number of factors, of which the "ripeness" of the milk is one of the most important. A longer time of manufacture means a longer period during which bacterial growth may take place. The ripeness of the milk used in these experiments varied little from day to day and the kettle process usually took a little over two hours.

The contents of the kettle undergo a series of temperature

changes. The temperature of the milk when it is received and when it enters the kettle may vary considerably, even from kettle to kettle. In this country most of the milk for Swiss cheese manufacture is clarified before it enters the kettle. Most cheese makers clarify the milk at the temperature at which it is received, but some of the more careful makers clarify the milk at 18° to 24°C. (65° to 75°F.) and warm the milk if it is too cold. In these experiments the milk was clarified at about 21°C. (70°F.) and reached the kettle at about that temperature. Some cheese makers begin to warm the milk to the setting temperature as soon as the first milk enters the kettle; others wait until the kettle is practically full. Thus the milk is at temperatures between 15° to 21°C. and the setting temperature, 31° to 35°C., for a period which may range from fifteen minutes up to thirty minutes or more. The skim milk to be used for standardization of the milk may have stood even longer.

The setting temperature is usually about 33°C. (91.4°F.). When the milk has reached that temperature, the starters are added and then the rennet. The curd is cut about thirty minutes later. The time from cutting until starting to heat the curd and whey mixture will vary with ripeness of the milk used and with other conditions, but will average about fifty minutes. About thirty minutes are usually taken in raising the temperature to the cooking temperature which is usually about 53°C. (128°F.) and an average time of thirty minutes more elapses before the curd is dipped. The temperature of the curd at dipping is usually only slightly below the cooking temperature.

The starters used during these studies consisted of *Streptococcus thermophilus* (C₃ strain), *Lactobacillus casei* Bergey 1923 (*L. helveticum* Bergey 1927) (39a strain), or *L. bulgaricus* (Bergey 1927) (Ga strain), and *Propionibacterium Shermanii* (No. 62 strain). The C₃ and 39a (Ga) organisms were grown separately or together in sterilized milk or whey. The quantity of starters used was varied little and then only with variations in the milk. The No. 62 bacteria were grown in glucose peptone broth and approximately equal numbers were added to each cheese.

METHODS

A special technique for sampling the kettle contents was necessary, so that comparisons could be made between milk samples taken at the beginning of the process and curd and whey samples taken later. The curd and whey samples were taken after the kettle contents had been thoroughly stirred so that the curd particles had been uniformly distributed throughout the whey. A sterile 30- or 50-cc. beaker was used for sampling. The curd was allowed to settle; the supernatant whey was poured off into another sterile beaker and the curd was transferred to a 4½-inch mortar which had been previously steamed and then cooled to about 50°C. The curd was ground in the mortar for one minute. If the curd was too dry, a little of the whey was added. Then sodium citrate was added at the rate of 0.2 gram for each gram of curd (estimated) and the grinding was continued until a smooth, even paste was formed (Burkey, 1931). Then the decanted whey was poured in and the contents of the mortar were thoroughly stirred. A homogeneous, milk-like fluid resulted. One cubic centimeter was used for making further dilutions for plate counts or dilution counts or for direct microscopic counts. For making pH determinations the samples were taken from the kettle in the same manner; curd and whey were separated, but the curd was ground without the addition of sodium citrate. The decanted whey was returned to the curd and pH determinations were made on the mixture of whey and curd.

When slides were to be made for the direct microscopic count of bacteria, a 0.1 cc. dilution was made by adding 1 cc. of the kettle milk or of the ground curd-whey suspension to a 9-cc. alcohol blank. From this, 1:10 dilution slides were prepared by the usual Breed method. The alcohol for dilution blanks was prepared as follows: Equal parts of 95 per cent alcohol and of distilled water were mixed, phenolphthalein was added, and then 15 per cent KOH solution was added until the alcohol solution was a deep pink in color. If the pink color disappeared on the addition of milk or curd-whey to the alcohol blank, alkali was added to bring back the pink color. The preparations were

fixed in a 2 per cent solution of calcium chloride in 95 per cent alcohol and stained by Burke's modification of the Gram method.

In early experiments samples were taken from the kettle at frequent intervals during the manufacturing process, but results soon showed that three samples were sufficient to show the course of bacterial growth in the kettle; a sample of the milk from the full kettle after addition of the starters, a sample of the curd and whey mixture just prior to the start of heating up to the cooking temperature, and a sample of the curd and whey just before the curd was dipped out of the kettle and into the cheese hoop.

For plate counts of bacteria tomato milk-powder agar was used. Citric acid agar was used for plate counts of aerogenes-like bacteria, and deep cultures in yeast-extract-peptone-sodium lactate agar were used to determine numbers of lactate-fermenting bacteria. The deep-culture tubes were sealed with 1 inch of 3 per cent agar. These tube cultures were incubated for at least five weeks at 30°C. before a count was made.

The citric acid agar had the following composition:

Citric acid.....	2.0 grams
Sodium chloride.....	5.0 grams
Magnesium sulphate.....	0.2 gram
Potassium acid phosphate (K_2HPO_4).....	0.3 gram
Ammonium acid phosphate ($NH_4H_2PO_4$).....	1.0 gram
Washed agar (3 per cent).....	500 cc.
Distilled water.....	500 cc.
Sodium hydroxide to bring reaction to pH 7.	

RESULTS

Propionibacterium Shermanii (No. 62), counted in the deep tubes, were found in numbers varying from 5000 to 10,000 per cubic centimeter of the kettle milk. The numbers remained constant or decreased slightly during the processes in the kettle. The other lactate-fermenting bacteria increased slowly in numbers.

Streptococcus thermophilus (C_3) and *Lactobacillus casei* (39a), or *L. bulgaricus* (Ga), were counted by both the plate and the direct microscopic methods in many experiments and only by the microscopic method in other experiments. All of these kinds of

starter bacteria form chains when they increase in numbers. Consequently, an increase in numbers would be detected by the microscopic method, by which individual cells can be counted better than by the plate method, by which groups or clumps of bacteria are counted. It is true that the microscopic method does not entirely distinguish between living and dead cells. The counts reported, however, were made from preparations stained by the Gram method and only the Gram-positive cells of the starter bacteria were counted. Cells old enough to be Gram-negative were considered dead. Any marked increase in numbers as determined by this method must be considered significant,

TABLE 1

Plate counts of starter bacteria in the kettle contents (bacteria per cubic centimeter)

CHEESE NUMBER	KETTLE MILK PLUS STARTERS		AT START OF HEATING (COOKING)		BEFORE DIPPING	
	C ₂ (<i>Str. thermophilus</i>)	39a (<i>L. casei</i>)	C ₂	39a	C ₂	39a
1358	100,000	180,000	390,000	450,000	510,000	380,000
1372	200,000	400,000	240,000	230,000	1,200,000	60,000
1399-1	60,000	160,000	210,000	130,000	840,000	140,000
1445-1	140,000	1,060,000	980,000	730,000	3,290,000	620,000
1450	190,000	1,220,000	370,000	1,030,000	1,090,000	850,000
Average for 23 cheeses.....	338,000	632,000	878,000	499,000	2,440,000	367,000

while a small decrease may mean nothing more than the aging of some of the cells.

The plate counts of the two chief kinds of starter bacteria shown in table 1 were made at the time of the addition of the starters to the full kettle of milk, at the time the heat was turned on to raise the temperature to that of "cooking," and at the time the curd was ready to be dipped from the kettle into the hoop. Counts made during the manufacture of 5 different cheeses are given and also the average counts for 23 cheeses. Both the individual counts and the averages show:

1. That the *Str. thermophilus* (C₂) bacteria usually increase in number from the time they are added to the kettle milk until the

curd is dipped. The increase is likely to be more rapid in the period from the start of heating to dipping, because the higher temperature is favorable to this high-temperature coccus. When the temperature has reached 53°C., however, growth is probably inhibited, for experiments have shown that these cocci do not grow well at temperatures above 50° to 51°C.

2. That the *L. casei* (39a) bacteria do not increase in numbers in the kettle, but in fact usually decrease in numbers, especially after the heat has been applied.

In table 2 are shown direct microscopic counts of the two kinds of starter bacteria in the kettle contents, with results comparable

TABLE 2

Direct microscopic counts of starter bacteria in the kettle contents (bacteria per cubic centimeter)

CHEESE NUMBER	KETTLE MILK PLUS STARTERS		AT START OF HEATING (COOKING)		BEFORE DIPPING	
	C ₃	39a	C ₃	39a	C ₃	39a
1399	8,500,000	1,380,000	38,900,000	948,000	103,000,000	790,000
1358	1,470,000	1,470,000	2,880,000	2,530,000	6,150,000	2,750,000
1387-1	3,950,000	5,140,000	4,200,000	5,460,000	17,600,000	2,100,000
1401-1	2,050,000	5,770,000	2,920,000	1,100,000	8,530,000	790,000
1445-1	1,830,000	5,050,000	3,950,000	2,900,000	14,800,000	2,870,000
Average for 25 cheeses.....	3,690,000	2,200,000	5,860,000	2,200,000	13,000,000	1,830,000

to those obtained by the plate method. The figures for cheese No. 1399 have been included in the table to show the effect of the addition of a larger number than usual of active C₃ cocci to the kettle milk. The much heavier growth of C₃ cocci in the kettle would markedly influence the working of the curd and would cause a lower pH of the curd at dipping time and probably a faster drop in pH of the cheese during the first hours on the press. These abnormal results were not included in the average results from 25 cheeses.

The 39a strain of *L. casei* is not as active as a *L. bulgaricus* culture called "Ga" in which the lactic acid rod is grown with a mycoderm. It was thought that the more active "Ga" culture

might grow to a small extent in the kettle. Table 5 shows, however, that "Ga" made little or no increase in numbers in the kettle and often had decreased in numbers by the time of dipping. The "C₁" coccus when added with "Ga" grew in the same manner that it did when added with the 39a culture.

TABLE 3

Plate counts of gas and lactic bacteria in the kettle contents

CHEESE NUMBER	ORGANISM	KETTLE MILK	BEFORE COOKING	BEFORE DIPPING
1445-1	Lactic	7,000	70,000	60,000
1445	Lactic	10,500	70,000	35,000
1672-1	Lactic	250,000	1,050,000	1,000,000
1666-1	Gas	400	100	50
1638-1	Gas	16,000	7,700	3,000
1638	Gas	1,290,000	350,000	950,000
1672	Gas	13,800,000	13,400,000	51,000,000
Average 6 cheeses with low count...	Gas	8,400	7,833	4,840
Average 5 cheeses with high count...	Gas	6,390,000	4,800,000	18,600,000

TABLE 4

Direct microscopic counts of gas and lactic bacteria in the kettle contents

CHEESE NUMBER	ORGANISM	KETTLE MILK	BEFORE COOKING	BEFORE DIPPING
1450-1	Gas	1,450,000	720,000	447,000
1450	Gas	527,000	1,510,000	757,000
1445-1	Gas	1,390,000	None found	276,000
1380	Gas	2,290,000	5,230,000	2,840,000
1401	Lactic	10,300,000	37,000,000	None found
1409-1	Lactic	297,000	2,550,000	None found
1433	Lactic	80,300,000	54,500,000	68,700,000

If the milk used is of low bacterial count, the starter bacteria will predominate over other bacteria in the kettle. With a poor milk, however, either the lactic or the gas bacteria or both may develop enough to be harmful. In table 3 are shown plate counts and in table 4 direct microscopic counts of gas bacteria and lactic bacteria in the kettle contents. These results would indicate

that the numbers of gas bacteria remain about constant during the making process in the kettle unless the original number in the

TABLE 5

Direct microscopic counts of "Ga" and "C₁" starter bacteria in the kettle contents

CHEESE NUMBER	KETTLE MILK PLUS STARTERS		AT START OF HEATING (COOKING)		BEFORE DIPPING	
	C ₁	Ga (<i>L. bulgaricus</i>)	C ₁	Ga	C ₁	Ga
1491	1,000,000	100,000	688,000	138,000	7,700,000	170,000
1505-1	1,980,000	82,600	4,300,000	198,000	14,500,000	82,600
1505	1,070,000	171,000	1,900,000	316,000	11,300,000	164,000
1511-1	5,340,000	723,000	5,980,000	550,000	19,100,000	378,000
1511	5,300,000	959,000	4,150,000	641,000	19,300,000	470,000
Average for 6 cheeses.....	2,680,000	356,000	3,150,000	343,000	12,500,000	239,000

TABLE 6

Changes in pH of kettle contents

CHEESE	STARTERS*	pH VALUES OF KETTLE CONTENTS			
		Before setting	Start of cooking	Before dipping	Total drop in pH
Average of 15 cheeses	Normal	6.57	6.54	6.48	0.09
No. IA	Normal	6.54	6.53	6.51	0.03
No. IB	1 per cent <i>Str. lactis</i> †	6.47	6.38	6.29	0.18
No. IIA	Normal	6.61	6.59	6.54	0.07
No. IIB	1 per cent <i>Str. lactis</i>	6.58	6.45	6.35	0.23
No. IIIA	Normal	6.62	6.57	6.51	0.11
No. IIIB‡	None	6.62	6.58	6.56	0.06
No. IVA	Normal	6.67	6.61	6.53	0.14
No. IVB‡	None	6.67	6.64	6.58	0.09

* Normal starters consisted of *Str. thermophilus* (C₁) and *L. bulgaricus* (39a).

† One per cent *Str. lactis* starter was added in addition to the normal starters.

‡ Cheeses IIIB and IVB were made on a small scale.

kettle milk is large, as in cheese No. 1672 in table 3. Then an increase will take place in the kettle, with gassy cheese as a possible result. This cheese was, in fact, practically a "nissler"

when cut, with too many and too small eyes. The gas bacteria present were mostly of the *A. aerogenes* type.

The lactic bacteria (*Str. lactis*) are apparently able to grow during the early part of the kettle process but their growth is stopped by the cooking temperature.

Table 6 shows the changes in pH of the kettle contents under different conditions. It will be noted that when normal quantities of starter are added there is a slow and gradual decrease in pH during the processes in the kettle. Similar changes occur even when no starter bacteria are added. Under such conditions the numbers of bacteria and the amount of their growth during the kettle processes are so small that pH changes must be attributed largely to physico-chemical rather than bacteriological causes. The presence of large numbers of bacteria of the *Str. lactis* type, as in cheeses IB and IIB, caused a greater drop in pH due to the growth and fermentative activity of this organism. The growth in the kettle contents of large numbers of any other acid-forming bacterium would produce a similar effect.

DISCUSSION

An understanding of the behavior of the different bacteria in the kettle is of considerable practical importance to the cheesemaker. Of the starter bacteria apparently only *Str. thermophilus* makes a significant amount of growth in the kettle. Large numbers or long-continued growth of these thermophilic streptococci can affect the working of the curd in the kettle. For instance, the addition of the *Str. thermophilus* starter when the first milk enters the kettle, instead of after the kettle is full and its contents have been heated to the setting temperature, gives these streptococci longer to grow and makes the milk a little "riper." There are indications, too, that this earlier addition of streptococci helps hold down the growth of the undesirable gas bacteria present, just as the streptococci apparently inhibit the gas bacteria throughout the kettle processes and in the cheese on the press. A more active growth and larger number of the streptococci in the kettle means a more rapid development of acidity in the cheese on the press and in extreme cases may even

start acid development in the curd before the curd is dipped. The effect of *Str. thermophilus* on ripening of the milk will be discussed in more detail in a later paper.

It is known, of course, that large numbers of gas bacteria in the milk will harm the quality of the cheese, and that a few gas bacteria will cause no trouble. The results of the above experiments show that when small numbers of gas bacteria are present in the kettle milk little or no growth will take place, but that when large numbers are present in the kettle milk growth takes place. If the cheesemaker knows that large numbers of gas bacteria are present he will add his *Str. thermophilus* starter early, and will probably increase the amount of this starter. As a consequence he will not only have the restraining influence of the thermophilic streptococci on the gas bacteria but will tend to shorten the time of foreworking and hence the time that the gas bacteria can increase in numbers.

If large numbers of lactic bacteria (*Str. lactis*) are present in the kettle milk, the milk will be "riper," and the early processes in the kettle will be shortened accordingly, with less time for growth of the lactic organisms. The addition of *Str. thermophilus* starter in large amounts to such a milk, or addition of this starter early in the process would be contrary to reason.

The time of addition of the *Lactobacillus* starter would not seem to be important, because these bacteria apparently do not grow to any extent in the kettle.

SUMMARY

Bacterial counts and pH determinations were made on the kettle contents during the manufacture of Swiss cheese. Samples were taken after the addition of the starters, just before the heat was turned on for the cooking process and just before the curd was dipped. The following results were obtained:

1. *Streptococcus thermophilus* (C₄) usually increased in numbers during the whole process in the kettle, but the increase was usually more rapid during the latter part of the process when the temperature of the kettle contents was higher.

2. *Lactobacillus casei* (39a) or *bulgaricus* (Ga) did not increase

in numbers in the kettle and usually decreased in numbers by dipping time.

3. *Propionibacterium Shermanii* did not increase in numbers during the kettle processes and often decreased slightly. The other lactate-fermenting bacteria slowly increased in numbers.

4. Gas-forming bacteria of the colon-aerogenes group showed little or no increase in numbers in the kettle when there were comparatively few in the original kettle milk. When the gas bacteria were in large numbers they showed a marked increase in numbers during the processes in the kettle.

5. *Streptococcus lactis* or "lactic bacteria" usually increased in numbers in the first part of the kettle process, but were stopped by the cooking temperature.

6. The pH of the kettle contents decreased to some extent during the processes in the kettle. Under normal conditions these pH changes were apparently due to physico-chemical rather than bacteriological causes.

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THE GENETIC SIGNIFICANCE OF THE DISSOCIANTS OF *STAPHYLOCOCCUS AUREUS*¹

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In the study of bacterial dissociation variations in pigment formation and in colonial and biochemical characteristics have been noted many times. As early as 1897, Newman reported the recovery of white colonies, differing in acid and indol production, from a strain of *Staphylococcus aureus*. Mellon and Caldwell (1926), studying a white strain of *Staphylococcus*, concluded that it was a common ancestor of *Staphylococcus aureus* and *albus*. More recently Pinner and Voldrich (1932), in a detailed study of six strains of *Staphylococcus aureus*, reported the isolation of four white, one lemon yellow, and one rose variant. In the same year, the authors obtained 11 variants from a single strain of *Staphylococcus aureus*, and the cultural characteristics of four of these were reported.

The purpose of this work was to study the dissociation of different strains of *Staphylococcus aureus* and *albus* and to determine their genetic position and cultural stability.

EXPERIMENTAL

Two strains of *Staphylococcus albus* and four of *Staphylococcus aureus* were dissociated and the characteristics of their variants studied. All the strains, before dissociation was attempted, were plated repeatedly on agar plates to insure a pure culture. When purity of cultures had been established all strains and all variants obtained later from these strains were dissociated (Hoffstadt and Youmans, 1932) in lithium chloride broth, pH 7.8, and plain

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broth, pH 7.8. Daily transfers were made serially into these broth tubes for a period of sixty days and subcultures from each broth tube were made on agar plates at each transfer. Incubation was at 37°C. No variants were studied for cultural characteristics or further dissociation until purity of the culture was determined. Utmost care was taken to prevent contamination.

CULTURAL CHARACTERISTICS OF THE STRAINS

The two strains of *Staphylococcus albus* differed from each other in that strain No. 2 did not ferment glycerol, and differed from Bergey's (1930) species *Staphylococcus albus* in not fermenting mannitol; with the exception of the heavy white growth on agar slants they were like *Staphylococcus epidermidis* described by Bergey. Strains 5 and 6 of the orange Staphylococci had identical cultural characters, both fermenting mannitol, but not glycerol or salicin in which they differed from the American Type Culture Collection strain as described by Pinner (1932). Bergey's species of *Staphylococcus aureus* does not ferment mannitol. Strain 3 orange agreed more nearly than any form with the latter, but differed from the American Type Culture Collection strain in that it did not ferment glycerol. Strain 4 showed the greatest variation; it did not ferment lactose, levulose, salicin, galactose or glycerol (table 1).

DISSOCIATION OF THE TYPES STUDIED

No. 1 of the white staphylococci showed no dissociation at any time, while from No. 2, the G, the gonidial form, was recovered on the thirty-ninth transfer. This strain of G fermented glucose, maltose, levulose, and lactose only feebly. Variants were isolated from all of the orange forms. From No. 3, a rough yellow was obtained on the forty-third transfer in lithium chloride broth, and from the forty-second transfer in plain broth, but it did not remain stable. A smooth, white stable variant and a translucent stable form were isolated on the sixth transfer in lithium chloride broth and from it on the fifty-first transfer, a yellow form was obtained, but no G forms were found.

The dissociation in strain 4, as previously reported by us (1932)

TABLE 1
Cultural characteristics of undissociated strains of staphylococci

SPECIES	ALBUS 1	ALBUS 2	AUREUS 3	AUREUS 4	AUREUS 5	AUREUS 6
Source*	Acne abscess	Acne abscess	Mild abscess	Abscess with osteomyelitis	Osteomyelitis (fatal)	Severe abscess
Pigment.....	White	White	Orange	Orange	Orange	Orange
Virulence.....	-	-	-	++++	++++	++++
Agar slant.....	Smooth white	Smooth white	Smooth yellow	Smooth yellow	Smooth yellow	Smooth yellow
Maltose.....	+	+	+	+	+	+
Mannitol.....	-	-	-	-	-	-
Inulin.....	-	-	+	+	+	+
Sucrose.....	+	+	+	+	+	+
Glucose.....	+	+	+	+	+	+
Levulose.....	+	+	-	-	+	+
Lactose.....	+	+	+	-	+	+
Salicin.....	-	-	-	-	-	-
Galactose.....	+	+	+	-	+	+
Glycerol.....	+	-	-	-	-	-

* From purulent discharges with exception of strains 4 and 5 which were from blood cultures.

was again confirmed with the production of two stable, smooth, white variants (SW), a rough yellow (RY), a rough white (RW), a translucent (SR), and the G form. In addition, a yellow smooth form was recovered from the smooth white on the twentieth transfer. The fifth strain never produced rough colonies, but light yellow smooth colonies appeared on the thirty-seventh transfer and became stable white colonies on the fifty-third. The G colonies were recovered twice from the forty-ninth transfer on lithium chloride broth and on the fifty-fifth in plain broth. Both the G colonies showed feeble fermentative powers. A yellow colony was obtained from the white variant on the fifty-first transfer. The variants of strain 6 were three in number: RY obtained in plain broth on the twenty-sixth day, but which did not remain stable; a smooth white on the thirty-fourth day; and a G form on the thirty-eighth, the forty-first and forty-third day in lithium chloride broth and on the twenty-ninth day in plain broth. The G forms fermented actively maltose, mannitol, sucrose, levulose and glucose. A yellow variant was recovered from the smooth white variant on the sixth day.

All the forms, except one, which showed dissociation were pigment producers and all gave rise to white stable strains. Three, strains 3, 4, and 6, dissociated into the G strain. Strain 4 produced the largest number of variants, and strain 3 only one variant, a feebly fermenting G. A stable rough form was recovered only from strain 4.

CHANGES IN THE CULTURAL CHARACTERISTICS OF THE WHITE VARIANTS OF ORANGE STAPHYLOCOCCI

Eight stable white variants were studied for cultural characteristics: two were isolated from culture 3, four from culture 4 and one each from cultures 5 and 6. They fell into two groups, those which fermented mannitol and those which did not (table 2). The former could again be subdivided into those which were glycerol-positive and those which were glycerol-negative. In the group which fermented both glycerol and mannitol, 3a and 4a were alike; however, 3a fermented both mannitol and levulose which the source, strain 3, did not. White 6a differed from the

TABLE 2
es of white variants of orange *id.*
Cultural char

	3a	3b	4SW	4SW ₂	4SR	4RW	5a	6a
Virulence.....	-	-	-	-	-	-	+++	-
Agar slant.....	Opaque	Translucent	Opaque	Translucent	Opaque	Opaque	Opaque	Opaque
Source.....	3	3	4	4	4	4	5	6
Maltose.....	+	+	+	+	-	+	+	+
Mannitol.....	+	-	+	-	-	+	+	+
Inulin.....	-	-	-	-	-	-	-	-
Sucrose.....	+	+	+	+	+	+	+	+
Glucose.....	+	+	+	+	+	+	+	+
Levulose.....	+	-	+	+	+	+	+	+
Lactose.....	+	+	-	+	+	+	+	+
Salicin.....	-	-	-	-	-	-	-	-
Galactose.....	+	-	-	-	+	+	+	+
Glycerol.....	+	-	-	-	-	-	-	+
Changes from original yellow culture	Mannitol + Levulose + Glycerol +	Galactose--	Levu- lose +	Mannitol - Lactose +	Maltose - Mannitol - Levulose + Lactose +	Levulose + Lactose + Galactose +	Pigment forma- tion only	Glycer- ol +

biochemical reactions of its parent culture only in its fermentative action on glycerol. Of those which did ferment glycerol and mannitol, 4 RW and 5a were alike while the third, 4 SW differed only in not fermenting lactose. It is interesting to note that 5a was the only white strain which did not lose its virulence after dissociation and that it differed from the parent culture only in pigment production; 4 SW differed from the original in the addition of levulose fermenting power and 4 RW in acid formation in lactose and galactose.

Only one form, 4 SW₂ agrees in fermentation reactions with any form described by Bergey, *Staphylococcus epidermidis*, but it was not in the least similar to it colonially. Not a single strain showed the fermentation reactions described by Bergey for *Staphylococcus albus* or *pharyngis*.

CULTURAL CHARACTERISTICS OF THE ORANGE VARIANTS

Five orange variants were obtained, one from strain 4 directly, a rough yellow, and one each from white strains 3b, 4 SW, 5a, and 6a. 5b and 6b were similar to each other, except that 5a fermented galactose, while all of the others differed in one or more fermentations. All differed from the white variant from which they were obtained, and from the original source culture (table 3).

SUMMARY AND DISCUSSION

All of the orange strains of staphylococci dissociated to some degree. Only one white strain showed any change, producing a feebly fermenting G form. White variants were recovered from all orange strains and G cultures from all but strain No. 3. Apparently the biochemical activity and the frequency of the appearance of the G form depend on the stability of the parent culture.

The progressive fermentative powers of the variants of strain No. 4, the most actively dissociating organism, are shown in table 4. The original cultural characteristics of this strain differed from all cultures of yellow and orange staphylococci described in that it did not ferment lactose. Of the first two variants isolated in its dissociation, 4 SW lost its pigment and like its parent did not

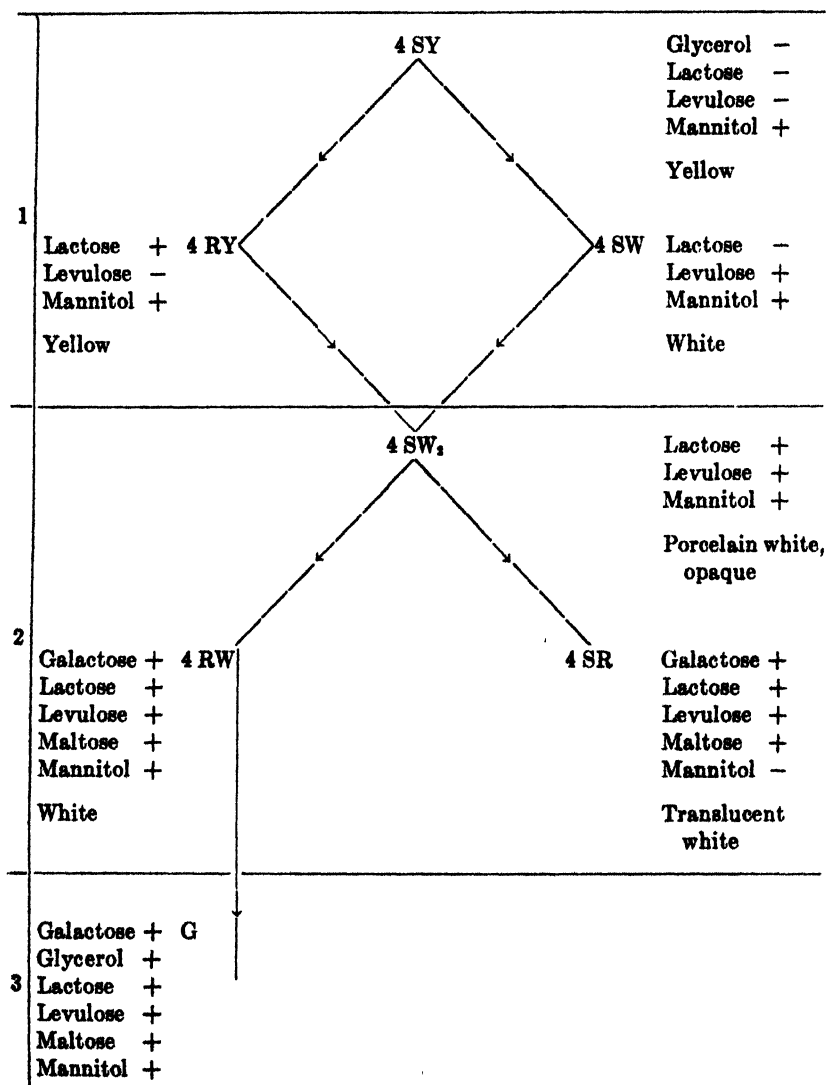
TABLE 3
Cultural characteristics of yellow

	3c	4a	4RY	5b	6b
Virulence.....	-	-	-	-	-
Source.....	3a	4SW ₂	4	5a	6a
Maltose.....	+	+	+	+	+
Mannitol.....	+	+	+	+	+
Inulin.....	-	-	-	-	-
Sucrose.....	+	+	+	+	+
Glucose.....	+	+	+	+	+
Levulose.....	+	-	-	+	+
Lactose.....	+	+	+	+	+
Salicin.....	-	-	-	-	-
Galactose.....	+	+	-	-	-
Glycerol.....	+	-	-	-	-
Changes from source form	Levulose +	Mannitol + Levulose + Galactose -	Lactose +	Galactose -	Galactose - Glycerol -
Changes from original yellow form.....	Mannitol + Levulose + Salicin + Glycerol +	Lactose +	Lactose +	Pigment galactose -	Galactose -

ferment lactose, but did ferment levulose, the other 4 RY, rough yellow, retained its pigment and fermented lactose, but not levulose. The next variant 4 SW₁, white, fermented both

TABLE 4

Distinguishing cultural characteristics of variants of strain 4 orange staphylococcus



lactose and levulose and although it did not ferment mannitol, produced 4 RW, rough white, which fermented mannitol, and 4 SR, translucent, white, which did not ferment mannitol. Both of these also fermented galactose and the G form, the last dissociant, fermented glycerol in addition. The loss of virulence and pigment production accompanied the change in fermentative activity. This was confirmed in all orange strains studied by Pinner and in all strains used in this study with the exception of strain 5 which did not lose its virulence and did not change its cultural characteristics when the pigment was lost.

It is not an unknown phenomenon in biological evolution for a biological variant to become a stable species. The evolution of the strain 4 variants with increasing fermentative powers has been divided into three sections (table 4). The first includes the establishment of lactose and levulose fermentation; the second includes the addition of galactose, and the third the addition of glycerol. Examining the cultural characteristics of the orange strains described by us, numbers 5 and 6, those possessing the greatest virulence, fall in section 1. In cultural characteristics the American Type Culture Collection strain falls low in this division and *Staphylococcus aureus* as described by Bergey falls at its juncture with section 2. The white variants fall within range 2 and 3, while *Staphylococcus epidermidis* as described by Bergey is nearest in cultural characteristics to the blind branch 4 SR₂ in section 2, and *Staphylococcus albus* and *pharyngis* in range 3.

Might not the 4 RY be the parent of the *Staphylococcus aureus* and 4 RW of *Staphylococcus albus*? Roughness is a relative term when applied to colonial form for many rough colonies may have a smooth appearance on ordinary media; hence a well established smooth-rough form may never appear colonially rough. This may account for Pinner's statement that he obtained no rough forms. We realize that the number of strains used is small, and in order to clear up the actual number of species within the staphylococcus group many more should be studied. However, if we are going to continue to use cultural characteristics in the classification of bacteria, the place of established dissociants, variants

and cultures which have the power of dissociation will have to be established.

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AN ACCESSORY FACTOR FOR LEGUME NODULE BACTERIA

SOURCES AND ACTIVITY

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The medium commonly used for the growing of legume nodule bacteria consists of the usual mineral salts, a source of energy (usually sucrose, mannitol, or glucose), and a water extract of yeast. In many experimental studies, where the addition of a material containing various forms of nitrogen and miscellaneous unknown substances is undesirable, nitrate, ammonia, or asparagine is substituted for the yeast water. Results vary widely as to how satisfactory this latter type of medium is, some investigators having reported heavy growths while others have obtained none. In the earlier work at this laboratory just as widely varying results were sometimes obtained when working with the same strain of organism and under supposedly similar conditions, but on different dates. The writers have determined the primary cause of most of these discordant results, namely, that many species of these organisms are unable to make an appreciable growth on a synthetic (sugar-mineral-nitrate) medium prepared from highly purified chemicals, but require an additional factor, probably organic in nature. This factor, as pointed out in a previous paper (Allison, Hoover and Burk, 1933), is a coenzyme directly essential for respiration and growth and not identical with "bios." For convenience it was termed coenzyme R.

The purpose of this paper is to present some of the earlier observations with typical data which led to the finding of the co-

¹ The writers are indebted to Drs. Dean Burk, G. E. Hilbert and C. A. Ludwig for numerous suggestions and helpful criticisms.

enzyme, and then to consider sources and preparation of the material in more concentrated form. Since the observations were made over a period of years, and in the earlier work were merely chance observations, some of the results may appear to be somewhat disconnected. It was, however, the accumulation of these miscellaneous observations that served as a basis for the later experiments and determination of the nature of the active factor. The previous paper stressed respiration but in this paper the results are reported in terms of growth. It should, however, constantly be borne in mind that the coenzyme is necessarily a growth factor as well as a respiration factor since anything essential for respiration must necessarily be essential for growth.

METHODS

Medium. The basal medium used in nearly all of the work was similar to the one recommended by Burk, et al. (1932) for *Azotobacter*. The composition of the medium was as follows: K_2HPO_4 , 0.7 gram, KH_2PO_4 , 0.3 gram; NaCl, 0.2 gram; $MgSO_4 \cdot 7H_2O$, 0.2 gram; $CaSO_4 \cdot 2H_2O$, 0.1 gram; and H_2O , distilled, 1000 grams. The addition of 0.01 gram $Fe(SO_4)_3 \cdot 9H_2O$ will sometimes improve it but numerous tests showed that the constituents of the medium usually contain enough iron impurity to supply the limited demands of the legume bacteria. The above stock solution is allowed to stand for at least two days, filtered, or the clear solution siphoned off, 1 per cent sucrose (or mannitol or glucose) added and sufficient KNO_3 to give 5 mgm. N per 25 cc. of the medium. Since this medium is a saturated solution of certain salts, the addition of 10 per cent additional water is necessary before autoclaving in order that the medium may remain clear after cooling. While this medium is excellent for *Azotobacter*, little or no growth of most rhizobia will occur in it if the sugar used is very pure, except where a heavy inoculum is used. The addition of the necessary respiration coenzyme, as will be shown later, causes a heavy growth. This medium, due to its clearness, proper pH (about 6.9), adequate buffer capacity, and synthetic composition is the most satisfactory the authors

have found for general laboratory use for either rhizobia (with coenzyme) or *Azotobacter* (without coenzyme).

Culture method. The usual method of studying the growth in these investigations was to maintain the bacteria at 28°C. in 250-cc. Erlenmeyer flasks, plugged with cotton and containing 25 cc. of medium per flask. Unless otherwise stated, two drops of a two- to five-day culture of the organism, grown on the above basal medium containing 1 per cent commercial sucrose, were used for inoculation. Tests were usually made in duplicate.

Organisms used. In the experimental work reported in this paper the clover organism, *Rhizobium trifolii*, strain 205, obtained from Dr. E. B. Fred of the University of Wisconsin, was the usual test organism used. *Rhiz. leguminosarum* (pea 302) and *Rhiz. meliloti* (alfalfa) were used in two experiments in comparison with clover 205. While no data are reported here for *Rhiz. phaseoli* (kidney bean), this organism was used in several experiments and gave responses practically identical with those obtained with the clover organism. These cultures had been tested frequently for purity and for ability to produce nodules.

Estimation of growth. All growth measurements reported here, except in table 1, represent bacterial numbers determined by direct microscopic count using a hemacytometer modified for bacterial counting. If the medium used is clear and the counts are made after a period of two to five days, while the organisms are growing rapidly and before clumping and film formation have become appreciable, the method is remarkably accurate. Successive counts, made on the same culture by a well-trained operator, seldom vary more than ± 5 per cent. The accuracy and speed are greater if the cultures are kept agitated during growth. This is not essential, however, but it is at least very desirable that all cultures be shaken a little every day during the growth period, as well as just prior to counting, in order to break up clumps. As many determinations as desired can be made on the same culture without interfering in the least with its growth. The work involved represents only a fraction of that required for plate counts, the accuracy is several times greater, and the result can be had at once instead of five days later. A comparison of

the method with certain other methods of measuring growth, such as by turbidity measurements, dry-weight determinations, and nitrogen analyses of the cells, only serves to emphasize the many advantages of the direct count method except for very special problems.

SOURCE AND QUANTITY OF INOCULUM

One of the first observations made at this laboratory, when investigations with legume nodule bacteria were begun a number of years ago, was that the quantity of growth obtained on a synthetic (sugar-mineral-nitrate) medium is markedly influenced by the source of inoculum, that is, the previous history of the organism, especially whether grown on a medium favoring a heavy or a very light growth. If cultures to be used later for inoculation were kept on a medium containing extracts of higher plants, yeast, or many other organic mixtures those organisms usually made at least a fair growth when transferred to a synthetic medium. In contrast, organisms that had previously grown under much less favorable conditions usually made little or no growth when placed in this medium. This observation was made so many times and the details so frequently checked that the earlier standard laboratory procedure followed for most experimental work was to transfer the stock cultures several times on yeast or other plant extract media just prior to their use. At the time it was believed that this procedure in some way modified the growth of the organism or possibly even affected its life cycle to such an extent that the effect continued for some time after being placed in an unfavorable medium. This explanation for the observed facts, as will be brought out more clearly later, was essentially wrong. The organisms grown on a rich medium contained such an abundance of the essential respiration and growth factor that when transferred, especially in large numbers, to a medium nearly free from this factor they were still able to grow. While the inoculation of such media with organisms taken from synthetic media resulted in little growth, the addition of a little yeast extract would cause excellent growth, showing that the organisms were alive and normal.

EGG ALBUMEN AS A GROWTH STIMULATOR

In the earlier stages of this investigation the effect on the growth of the addition of various materials, particularly plant extracts and other organic substances, to synthetic media was studied (Allison, 1927). Among the many materials tested was commercial egg albumen. The addition of 0.05 to 1.0 per cent of this

TABLE 1
Growth of legume bacteria on media containing egg albumen

MATERIAL ADDED	QUANTITY ALBUMEN ADDED	QUANTITY KNO ₃ ADDED	GROWTH OBSERVATIONS AFTER 7 DAYS	
			<i>Rhiz. trifolii</i>	<i>Rhiz. legu- minosarum</i>
	per cent	mgm. N per 25 cc.		
Check.....	0	4	+	+
Commercial egg albumen.....	0.1	4	++++	+++
	0.2	4	+++++	++++
	0.2	0	+	+
Pure egg albumen.....	0.1	4	+	+
	0.2	4	++	++
Commercial egg albumen, hydrolyzed	0.1	4	++++	+++
	0.2	4	+++++	++++
Pure egg albumen, hydrolyzed . . .	0.1	4	+	+
	0.2	4	++	++
Commercial egg albumen, hydrolyzed.	0.1	0	++++	+++
	0.2	0	+++++	++++
Pure egg albumen, hydrolyzed.....	0.1	0	+	+

* The number of +'s denotes relative growth.

material to a sugar-mineral-nitrate medium causes the formation of a heavy growth of the clover bacteria within a period as short as two or three days. Table 1 shows a typical series of results. In this experiment glucose was used as the source of energy and both the clover and pea bacteria as test organisms. This experiment was carried out before the system of direct microscopic

counting was in general use at this laboratory, hence the results are reported as relative growths as observed by the eye. Where a clear medium is used this method, as we have since determined, checks rather closely with actual bacterial counts and for many purposes is adequate.

The pure egg albumen had only a slight stimulating effect compared with that of the unpurified sample, thus furnishing additional proof that the impurities present and not the albumen itself are important in this connection. The sample of purified crystalline egg albumen was prepared by Dr. D. B. Dill of Harvard University.

The effect of hydrolysis is also shown in table 1. Samples of both the commercial and purified egg albumen were hydrolyzed by boiling in 25 per cent (by weight) of H_2SO_4 for twenty-six hours with reflux condenser attached. The stimulating factor was not appreciably affected by this drastic treatment. Furthermore, the hydrolyzed commercial albumen produced a heavy growth in the absence of KNO_3 , the amino acids serving as excellent nitrogen sources. The hydrolyzed pure egg albumen produced only a fair growth.

At the time these experiments were carried out the nature of the growth stimulant was not known but the experiments left little doubt that such a factor was present in the egg albumen as an impurity. It was also established that its activity could not be attributed to its value as a nitrogen or energy source and that it was highly resistant to acids.

In recent quantitative experiments, carried out for the purpose of checking the results reported in table 1, hydrolyzed commercial egg albumen produced a slightly better growth than did the equivalent weight of unhydrolyzed material. Furthermore, the activity of extracts (described on a subsequent page), containing the factor in concentrated form, was not affected appreciably by the acid hydrolysis.

A COMPARISON OF ENERGY SOURCES

Another entirely different line of evidence was obtained from numerous studies with various carbohydrates. In the earlier

tests of energy sources in media containing plant extracts it was found that glucose, sucrose, and mannitol were about equally good. Glucose was chosen for general use. In subsequent experiments, using synthetic media, sucrose in many cases gave much better results. It was thought, at first, that the formation of toxic compounds from glucose during the sterilization process was responsible for the poor growth but sterilization at lower pressures did not result in as good growths as with the sucrose. Sterilization does, of course, break down glucose to acids as Smith (1932) has shown, and these acids may be harmful to growth but this was not the major factor involved in this case.

TABLE 2

The stimulating effect of commercial egg albumen and natural humic acid on Rhiz. trifolii, using different energy sources

MATERIAL ADDED	1 PER CENT GLUCOSE		1 PER CENT COMMERCIAL SUCROSE		
	5 days	7 days	2 days	5 days	8 days
	millions per cc.	millions per cc.	millions per cc.	millions per cc.	millions per cc.
Check..	2	10	60	400	840
Natural humic acid, 10 p.p.m.			156	780	1,000
Natural humic acid, 25 p.p.m.	20	100			
Natural humic acid, 100 p.p.m.	180	300	320	940	1,380
Natural humic acid, 1,000 p.p.m.			500	2,000	2,500
Egg albumen, 200 p.p.m.			196	800	1,280
Egg albumen, 2,000 p.p.m.	1,000	2,000	420	2,100	2,000
Egg albumen, 20,000 p.p.m.			1,060	4,600	5,000

The data in table 2 show the importance of the sugar source. At the end of a growth period of five days in a glucose-nitrate medium there were 2 millions of clover bacteria per cubic centimeter while with commercial sucrose there were 400 millions present. The figures can not be considered as direct comparisons in this case because the experiment with sucrose was carried out a few days later than that with glucose but under the same experimental conditions. Such results have been obtained frequently and illustrate the point mentioned previously, that growth on a sugar-mineral-nitrate medium may vary from practically nothing to a very heavy one.

It is of interest to note that with glucose as the energy source heavy growths were obtained where either natural humic acid or egg albumen was present, showing that the glucose is actually a good energy source for clover bacteria and the failure to use it in the absence of the humic acid or egg albumen can be attributed to the absence of a necessary constituent in the medium. The growth with commercial sucrose was somewhat more rapid but both energy sources gave heavy growths where the medium was satisfactory.

Humic acid, prepared from soil, served as a good source of the growth factor although at the concentration used the growth was

TABLE 3
A study of various energy sources for Rhiz. trifolii

ENERGY SOURCE	BACTERIAL NUMBERS AFTER 4 DAYS WITH VARYING CARBOHYDRATE CONCENTRATIONS			
	0.5 per cent	1 per cent	2 per cent	4 per cent
	millions per cc.	millions per cc.	millions per cc.	millions per cc.
Glucose, C.P.....	22	10	No growth	No growth
Sucrose no. 1, C.P.....		60	60	60
Sucrose no. 2, C.P.....		70	80	120
Sucrose no. 3, commercial.....		360	620	640
Mannitol, C.P.....		30	30	36
Mannitol, C.P. with yeast water.....		840		

not as good as with egg albumen. A discussion of natural humic acid and other active iron compounds in relation to the growth of nodule bacteria will be reserved for inclusion in a later paper.

In table 3 are data giving a direct comparison of growth on a synthetic medium using glucose, mannitol, and three samples of sucrose as the energy sources. Samples of pure sucrose, as well as of glucose and mannitol, produced relatively small growths while commercial sucrose produced a heavy growth. Obviously, then, it is the impurity in the sugar that is important and not the particular energy source. Highly purified sucrose is probably little if any better as a source of energy than is pure glucose or mannitol. In this particular experiment the results

with glucose may be set aside since the flasks were sterilized at 15 pounds pressure for fifteen minutes and evidently toxic compounds were produced. The growth on the synthetic medium containing mannitol was very limited but with 10 per cent yeast water added a heavy growth was obtained, showing that mannitol is an excellent energy source but is used only if the bacterial growth factor is also present.

The data of table 3 also indicate that with the possible exception of the medium containing yeast extract in no case was sufficient of the growth essential present for maximum growth. Sucrose sample 1 and the mannitol were evidently quite free from the growth factor since increasing concentrations gave practically no increase in growth. Sucrose sample 2, although labelled C.P., contained a little of the factor because growth did increase with increase in quantity used. With such slight growths 0.1 per cent sugar would have been more than adequate to meet the energy requirements for a four-day growth period, as will be shown later in table 4, hence the increased growth can not be attributed to the sugar itself. One per cent commercial sucrose did not furnish enough of the growth factor for maximum growth since doubling the concentration nearly doubled the growth, but it is evident that it contained a high concentration compared with that in the C.P. sucrose. With 2 per cent commercial sucrose the growth was about 75 per cent as good as in the usual standard 1 per cent mannitol yeast extract medium.

It will be observed further that 60 millions of bacteria per cubic centimeter developed in four days in the cultures receiving sucrose sample 1 even though the sugar was practically free from the essential respiration and growth factor. The culture used for inoculation was grown on a commercial sucrose medium and the 2 drops of inoculum carried enough of the growth factor to permit the limited development observed. The same explanation holds for most of the results reported in subsequent tables.

Additional results, showing the growth of the clover nodule bacteria on various concentrations of pure and commercial sucrose, are given in table 4. It will be observed again that the growth was not appreciably affected by the concentration of pure

sucrose used, while with the commercial sample the growth increased markedly and rather uniformly with concentration.

Sugar analyses made by the method of Stiles, Peterson and Fred (1926), show that even with the lowest concentrations added the percentage present at the end of seventeen days was still greater than the quantity consumed. Lack of energy, therefore, was not the primary cause of limited growth in any of the flasks, although there was undoubtedly some effect due to concentration.

Table 5 shows the comparative activity of 4 samples of com-

TABLE 4

The effect of various concentrations of sugars on the growth of Rhiz. trifolii

SOURCE OF SUGAR	SUGAR ADDED	BACTERIAL NUMBERS AFTER			SUCROSE	
		3 days	6 days	14 days	Present after 17 days	Con- sumed in 17 days
	<i>per cent</i>	<i>millions per cc.</i>	<i>millions per cc.</i>	<i>millions per cc.</i>	<i>per cent</i>	<i>per cent</i>
Glucose, C.P.....	1	20	60			
	0.2	20	56			
	0.5	44	80			
Sucrose no. 1, C.P.....	1	48	100			
	2	44	72			
	5	34	76			
Sucrose, no. 3, commercial....	0.2	90	160	220	0.12	0.08
	0.5	132	240	360	0.38	0.12
	1	152	400	600	0.76	0.24
	2	180	600	1,500	1.55	0.45
	5	240	720	1,600	3.57	1.43

mercial cane sugar, all being quite active in comparison with pure sucrose. None of the sugars, however, contained sufficient coenzyme to allow of near-maximum growth where used at the usual 1 per cent concentration. The rather heavy inoculum used is again the explanation for the amount of growth observed with the pure sucrose.

PURIFICATION OF SUCROSE BY RECRYSTALLIZATION

A sample of commercial sucrose 3, which gave excellent growths in the above experiments, was purified by crystallization from

hot 30 per cent alcohol followed by recrystallization from hot 70 per cent alcohol. These two samples were then tested in com-

TABLE 5

A comparison of various samples of commercial sucrose on the growth of Rhiz. trifolii

	PER CENT	BACTERIAL NUMBERS		
		3 days	4 days	7 days
		millions per cc.	millions per cc.	millions per cc.
Sucrose no. 1, C.P.	0.5	36	40	100
	1.0	30	46	80
Sucrose no. 3, commercial	0.5	80	175	180
	1.0	100	360	360
Sucrose no. 4, commercial	0.5		100	
	1.0		220	
Sucrose no. 5, commercial...	0.5	100	150	200
	1.0	130		340
Sucrose no. 6, commercial...	0.5	80	150	220
	1.0	106		300

TABLE 6

Growth tests with sucrose purified by recrystallization from alcohol, using Rhiz. trifolii

SOURCE OF SUCROSE	BACTERIAL NUMBERS WITH VARYING SUCROSE CONCENTRATIONS									
	3 days					5 days				
	0.2 per cent	0.5 per cent	1 per cent	2 per cent	3 per cent	0.2 per cent	0.5 per cent	1 per cent	2 per cent	3 per cent
	mil- lions per cc.	mil- lions per cc.	mil- lions per cc.	mil- lions per cc.	mil- lions per cc.	mil- lions per cc.	mil- lions per cc.	mil- lions per cc.	mil- lions per cc.	mil- lions per cc.
Sucrose no. 1, C.P.	30	34	34	38	24	44	50	50	50	36
Sucrose no. 3, commercial	46	86	170	220	280	82	200	390	640	720
Sucrose no. 3, 1st recrystallization.	34	50	48	48	20	54	90	92	94	170
Sucrose no. 3, 2nd recrystallization.	14	*	*	18	20	26	*	*	60	124

* Lost.

parison with some of the original commercial sucrose as well as with C.P. sucrose. The results are shown in table 6.

It will be observed that one crystallization from alcohol removed most of the active material from the sugar; after the second crystallization the final product was nearly as free from activity as was the C.P. sucrose.

PREPARATION OF ACTIVE EXTRACTS FROM SUCROSE

Attention was next turned toward the preparation of the active factor in a concentrated form. A 50 gram sample of commercial sucrose no. 3 was extracted in a Soxhlet extractor with 100 cc. of absolute alcohol for about fifteen hours. The extract was evaporated to dryness under partial pressure at 50°C., taken

TABLE 7

Growth tests with an absolute alcohol extract prepared from commercial sucrose, using Rhiz. trifolii

TREATMENT	QUANTITY ADDED	BACTERIAL NUMBERS AFTER 4 DAYS
	<i>per cent</i>	<i>millions per cc.</i>
Sucrose no. 1, C.P.....	1	84
Sucrose no. 3, commercial.....	1	480
Sucrose no. 1 (1 per cent) + alcohol extract. . .	0.25*	140
	0.5*	300
	1*	420
	2*	660
Sucrose no. 3, alcohol extracted . . .	1	120

* These figures refer to extract equivalent to quantity of sugar designated.

up with water and again evaporated to remove all traces of alcohol. The residue was taken up in 50 cc. of water, filtered to remove the small quantity of material which did not go into solution, and its activity tested as in previous experiments.

The data given in table 7 show that absolute alcohol removed nearly all of the growth factor and that the final aqueous extract exerted a stimulating effect only slightly less than that of the equivalent amount of original sucrose. This indicates that the impurity in the sugar is present largely on the surface of the crystals.

An improved method used in obtaining the active factor was as follows: Two 350-grams samples of commercial sucrose were

extracted in a large Soxhlet extractor for fifteen hours with absolute alcohol, the extracts combined, kept over night at about 0°C., filtered, and evaporated to dryness under partial pressure as previously. The residue weighed 1.72 mgm. per gram of sugar extracted. A portion of this alcohol extract was in turn extracted with benzene and the latter removed by evaporation. Table 8 gives the results of the tests of the alcohol and

TABLE 8

Growth tests on Rhiz. trifolii using absolute alcohol and benzene extracts prepared from commercial sucrose

TREATMENT	QUANTITY SUCROSE ADDED	QUANTITY EXTRACT ADDED		BACTERIAL NUMBERS 4 DAYS
		Dry matter	Sucrose no 3 equivalent	
	per cent	p p m.	grams	millions per cc.
Sucrose no. 1, C P.	1			36
	2			36
Sucrose no 3, commercial	1			260
	2			480
Sucrose no 3, extracted	1			44
	2			50
Sucrose no. 1 + alcohol extract	1	80	1.16	390
	1	160	2.33	620
	1	320	4.67	700
Sucrose no. 1 + benzene extract.....	1		1.16	36
	1		2.33	36
	1		4.67	36

benzene extracts, as well as of the original and extracted sugar, on the clover nodule bacteria.

The data show that the absolute alcohol extracted practically all of the active material from the cane sugar and that the extract, when added to C.P. sucrose, produced essentially the same effect as did the original sucrose. The addition of 80 p.p.m. dry weight of the extract to the medium containing C.P. sucrose produced nearly an 11-fold increase in bacterial numbers within a

growth period of four days, while larger additions produced even greater growth increases. The data also show that the factor is not soluble in benzene. An ether extract tested subsequently was also inactive.

A considerably more concentrated extract was then prepared by extracting about 4 kgm. of commercial sucrose (sample 5) in successive 350-gram portions in a large Soxhlet extractor for about five hours each, using the same alcohol for all extractions. This, of course, decreased the quantity of sugar per unit of active material present in the final extract. Table 9 gives the results of two separate tests of the active extract.

TABLE 9

Growth tests with active extract prepared from commercial sucrose, using Rhiz. trifolii

TREATMENT	BACTERIAL NUMBERS AFTER 4 DAYS	
	First ex- periment	Second ex- periment
	millions per cc.	millions per cc.
Sucrose no. 1, C.P. 1 per cent	26	18
Sucrose no. 1 + 7 p.p.m. dry matter in extract	106	200
Sucrose no. 1 + 18 p.p.m. dry matter in extract	320	450
Sucrose no. 1 + 36 p.p.m. dry matter in extract	580	
Sucrose no. 1 + 72 p.p.m. dry matter in extract	580	

As little as 7 p.p.m. of dry matter in the extract was sufficient to produce a 4-fold stimulation of bacterial growth after four days in one experiment and 11-fold in the other. Using 18 p.p.m. the corresponding figures were 12-fold and 25-fold. This extract, on analysis by the method of Stiles, Peterson and Fred (1926), was found to consist of 75 per cent sugar.

CANE MOLASSES AS A SOURCE OF GROWTH FACTOR

The above experiments show that the bacterial growth factor can be almost quantitatively removed from commercial sucrose by extraction with absolute alcohol. Since the factor is presumably present chiefly on the surface of the sucrose crystals the logical assumption is that it is contained in the film of molasses

always present in traces in commercial cane sugar. In table 10 are given data showing the activity of ordinary cane molasses.

Approximately 70 p.p.m. of the molasses, about one-third of which was water, gave a growth of half-maximum. In table 9, 18 p.p.m. of the most concentrated extract of sucrose produced a somewhat similar effect. The alcohol extract of sucrose was, therefore, approximately three times as concentrated in growth

TABLE 10

The growth-promoting properties of varying concentrations of cane molasses, using Rhiz. trifolii

TREATMENT	QUANTITY MOLASSES ADDED*	BACTERIAL NUMBERS AFTER 4 DAYS		
		Experi- ment 1	Experi- ment 2	Experi- ment 3
	<i>p p m.</i>	<i>millions per cc</i>	<i>millions per cc.</i>	<i>millions per cc</i>
Check (sucrose no. 1, c.p.)		50	28	26
	10,000	900		
	8,000	760		
	4,000	800		
	2,000	860	920	
	1,000	860	960	
	800		920	
Cane molasses	400		960	1,000
	200		700	800
	80		480	640
	40		320	280
	20			160
	8			80
	4			42
	2			30
	0 8			26

* These quantities refer to ordinary molasses of which approximately one-third consists of water.

factor per unit of dry matter as was the molasses. The crude molasses per unit dry weight, was nearly 200 times as active as commercial sucrose.

PREPARATION OF ACTIVE EXTRACTS FROM CANE MOLASSES

In order to prepare a concentrated extract from molasses it must first be thoroughly dried and ground. The most satis-

factory method found for accomplishing this was as follows: 25 grams of cane molasses were thoroughly mixed with 60 grams of sand (passing 30-mesh) which had previously been calcined, and dried under reduced pressure at 90°C., with a current of heated dried air passing through the system. The dried sand-molasses mixture was quickly ground in a mortar and extracted with absolute alcohol in a Soxhlet extractor for about six hours in the usual manner. The alcohol was then distilled off under reduced pressure and the activity of the dried residue compared with that of the extract previously prepared from sucrose (see table 9).

TABLE 11

A comparison of the growth-promoting properties of alcoholic extracts of sucrose and molasses on Rhiz. trifolii

QUANTITY OF EXTRACT ADDED p p m.	BACTERIAL NUMBERS AFTER 4 DAYS			
	First experiment		Second experiment	
	Sucrose extract	Molasses extract	Sucrose extract	Molasses extract
	millions per cc.	millions per cc.	millions per cc.	millions per cc.
0	10	10	24	24
1	16			
2	20			
4	30			
8	80			
16	140		320	380
32	300	440	460	560
64	500			
128	640			
160		800		

The activity per unit of dry matter of the molasses extract, as shown in table 11, was only approximately 30 per cent greater than that of a similar extract of sucrose even though the original molasses was nearly two hundred times as active as was the commercial sucrose. This is not surprising since the source of the material and method of extraction are essentially the same in both cases. A comparison of the data given in table 11 with those in table 10 also shows that the alcoholic extract of molasses was about four times as active as molasses itself, 16 p.p.m. of the extract giving a 16-fold increase in growth within four days or

nearly half the usual maximum for such an experiment. This extract still contained 78 per cent sugar. Further work on the preparation of more active extracts is now in progress.

COMPARISON OF YEAST EXTRACT WITH SUCROSE EXTRACT

It was pointed out previously that a sugar-mineral-yeast medium is used extensively for growing rhizobia. As a result of the studies of this laboratory it is now quite evident that the chief function of the yeast is to supply the necessary respiration factor to permit growth. If some other source of the coenzyme and nitrogen are supplied, then the yeast water may be omitted

TABLE 12

A comparison of the growths obtained with yeast water and the alcoholic extract of commercial sucrose using different nitrogen sources

TREATMENT (ADDITIONS TO BASAL MEDIUM)	BACTERIAL NUMBERS AFTER 3 DAYS	
	<i>Rhiz. trifolii</i>	<i>Rhiz. meliloti</i>
	millions per cc.	millions per cc.
Check (with KNO_3)	20	20
Yeast water 10 per cent + KNO_3	540	740
Yeast water 10 per cent + urea	420	400
Sucrose extract 40 p.p.m. + KNO_3	360	280
Sucrose extract 40 p.p.m. + urea	14	440
Sucrose extract 40 p.p.m. + NH_4Cl	500	360
Sucrose extract 40 p.p.m. + asparagine	340	600

from the medium without greatly affecting its value. Table 12 gives typical data, obtained many times, illustrating this point.

It will be observed that in most cases the yeast water gave a slightly better growth than did the alcoholic extract of sucrose but both preparations were excellent growth promoters and usually gave increases of 15- to 35-fold over the check. The actual increases in growth depended to a large extent upon the nitrogen source used. All four sources of nitrogen tested were utilized satisfactorily by the two legume organisms except in the case of urea which was toxic to the clover, but not to the alfalfa organism. A determination of the pH of the cultures on the

third day showed that where urea was added the pH was high, usually near 8. It was probably even higher on the first day. If smaller amounts of urea had been added it is probable that it would have been utilized satisfactorily by the clover as well as by the alfalfa organism. The results of this experiment check closely with the data reported in table 3 where a 2 per cent commercial sucrose-mineral-nitrate medium gave approximately 75 per cent as good growth in four days as the usual mannitol yeast water medium.

Direct comparisons of yeast water and sugar extracts almost invariably show that the yeast water produces a somewhat more rapid initial growth stimulation than does the sugar extract but at the end of four or five days the differences are much less marked. The fact that yeast extract is slightly more stimulating is not surprising since the yeast water contains in addition to the respiration factor a variety of sources of nitrogen, traces of mineral elements, and miscellaneous factors. The fact that the sucrose extract is ordinarily 60 to 90 per cent as good as the yeast water, in spite of the many other materials present in the latter, only serves to emphasize that it is the essential respiration factor in the yeast that is of primary importance.

DISCUSSION

The experiments reported here have shown rather conclusively that legume nodule bacteria, at least the clover, pea, and alfalfa species, require an accessory growth factor not found in appreciable quantities in highly purified sugars but present in commercial sucrose, cane molasses, commercial egg albumen and yeast. This factor, as already shown (Allison, Hoover and Burk, 1933), is a respiration coenzyme and exerts its effect on growth indirectly through its effect upon respiration. In the light of previous work of this laboratory, already published (Allison, 1927), it may also be stated that the respiration coenzyme is widely distributed in the plant kingdom, and probably also in the animal kingdom.

While the necessity for adding yeast extract or similar material to legume culture media in order to obtain abundant

growths has long been appreciated, practically no work has previously been reported dealing directly with the nature of the growth factor. Where the subject has been discussed the greatest emphasis has usually been placed upon the nitrogen compounds present in the yeast extract. Since it has been shown here that about 16 to 20 p.p.m. of an alcoholic extract of sucrose or molasses, still consisting chiefly of sugar, is sufficient to produce a heavy growth when added to a sugar-mineral-nitrate medium it is quite obvious that the nitrogen source is of secondary importance. It was pointed out above that legume bacteria, at least the species studied, are able to utilize readily the common simple nitrogenous compounds ordinarily supplied in bacterial culture media. There is, likewise, no evidence, so far as the writers have observed, that the carbohydrate or mineral requirements of these species are highly specialized.

In the light of the findings reported here it is interesting to search through the literature and note how many previously reported observations, heretofore inexplicable, can now be quite logically explained. Fred, Baldwin and McCoy (1932) mention several of these which need not be listed here. Many references have been made to the fact that legume bacteria grow more profusely on agar than in liquid culture media. This statement is not true for the clover, alfalfa, bean or pea bacteria if the medium is satisfactory, for certainly a growth of nearly one billion bacteria per cubic centimeter within four days, as commonly obtained with an adequate growth factor, is about as profuse a growth as most common soil organisms, such as *Azotobacter*, give. In the past, agar media were sometimes found to be superior to liquid synthetic media primarily for the reason that the latter frequently contained little coenzyme. Experiments at this laboratory have shown that agar commonly contains a fair amount of the essential factor. The observations of Allyn and Baldwin (1930) that not only dried agar but silica sand and ground filter paper favor the initiation of growth is also interesting. We have tested samples of quartz sand and found that even though supposedly rather pure it was possible in all cases to extract from the sand some of the growth factor which upon

addition to the culture medium produced a considerably increased growth, but, of course, only a fraction of the maximum. Extracts of soil, added in sufficient quantity, will give excellent growths.

SUMMARY

1. Experiments with legume nodule bacteria, *Rhiz. trifolii*, *Rhiz. leguminosarum*, and *Rhiz. meliloti*, are reported showing that these organisms are unable to make an appreciable growth in an ordinary synthetic medium containing highly purified sugars as the only energy source. Experiments, not reported in detail, showed that *Rhiz. phaseoli* behaves similarly.

2. The failure to grow in such a medium is due to the absence of a necessary factor, which, as previously pointed out, is a respiration coenzyme, essential primarily for respiration and indirectly for growth.

3. Studies reported here show that this respiration coenzyme is present in relatively high concentrations in yeast, cane molasses, humic acid and commercial egg albumen; commercial sucrose contains a smaller percentage. In fact, it is quite widely distributed in the plant kingdom and probably also in animal products.

4. The essential respiration and growth factor can be obtained readily in fairly concentrated form by extraction of commercial sucrose or dried cane molasses with absolute alcohol. Such extracts, even though containing about 75 per cent sugar, are of such activity that 16 to 20 p.p.m. added to a synthetic medium will commonly produce a half maximum growth.

5. Neither the nitrogen nor sugar requirements of *Rhiz. trifolii* are highly specific. The organism grows readily upon most of the common laboratory media used in soil bacteriological studies provided the pH is satisfactory and a source of the essential factor is supplied.

6. Direct comparisons of the growth of *Rhiz. trifolii* and *Rhiz. meliloti* in the commonly used sugar-mineral-yeast medium with that in a sugar-mineral-nitrate-coenzyme medium show that the growth in the latter is usually 60 to 90 per cent as good as in the former in spite of the miscellaneous substances in the yeast, par-

ticularly nitrogen and traces of various minerals in readily available form, known to favor growth.

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DISSOCIATION IN YEASTS¹

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INTRODUCTION

The instability of bacterial species has been noted by various workers in the field of bacteriology since bacteria became the subject of intensive study. As early as 1877 Nägeli pointed out the possibilities of such instability. He held the view that the fission fungi represented only a single type of cell which was highly sensitive to its environment and was capable of great variability as regards its morphological and physiological characteristics. At about the same time, while Nägeli and his colleagues were pointing out the instability of bacterial species, another group, headed by Cohn and Koch was insisting with equal certainty on the constancy of bacterial species. Almost from the very beginning of bacteriology, then, one finds two widely different views in regard to the stability of bacterial species. The more conservative views of Cohn and Koch prevailed so that there gradually was recognized for each bacterial species only one characteristic morphological and physiological type. However, from time to time different workers in bacteriology were observing variations in bacterial types too significant to be ignored. As the results of these observations were published, sufficient data were accumulated to convince the most skeptical that considerable variation does exist in bacterial species as regards their morphological, physiological and immunological characteristics. Hadley (1927)

¹ Journal article no. 169 from the Michigan Agricultural Experiment Station.

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has made a most excellent and extensive review of the subject in his paper on microbial dissociation.

DISSOCIATION IN YEASTS

When one turns from bacteria to yeasts, there does not appear to have been the same abundance of work on dissociation. In fact the whole question seems to have been neglected. However, there are certain data available which leave no doubt that considerable variation does exist in yeast species. Hansen (1895) has perhaps shown most clearly that yeasts may undergo important variations, some of which are permanent while others are transitory. In his work with *Saccharomyces carlsbergensis* he found that if this yeast was grown at 27°C., it produced normal cells with typical colonies. If, however, it was cultivated at 7°C., the cells were elongated, forming a sort of mycelium, and the colonial forms were very different from the normal colonies. He was able to preserve this variation for six months. Variations of this nature were considered temporary.

Lepeschkin (1903) in his work with yeasts, especially *Schizosaccharomyces mellacei*, found constant variations. When a young culture of *Schizosaccharomyces mellacei* was grown in glucose yeast water, there appeared mycelial forms, either with or without ascospores. He was able to maintain the mycelial forms, in pure culture indefinitely.

Guilliermond (1919) has likewise been able to obtain mycelial forms in a young culture of *Schizosaccharomyces pombe* and maintain them constantly in pure culture. With *Saccharomyces Ludwigii* he was able to isolate and maintain in pure culture three distinct forms, a sporogenic strain, an asporogenic strain and a feebly sporogenic strain.

Lindner (1909) cultivated *Saccharomyces bailii*, *Pichia hyalosporea* and *Pichia farinosa* for a long time on gelatin and found that they had lost their ability to produce spores. Holm, according to Guilliermond (1919), has been able to do the same thing with *Saccharomyces multispurus* by culturing it a long time on beer wort with sucrose.

Beijerinck (1897) in growing *Schizosaccharomyces octosporus*

on nutrient gelatin noticed three types of colonies: White colonies, the cells of which produced ascospores; light brown colonies, made up of a mixture of cells, some of which were asporogenic while others were sporogenic; and brown colonies containing cells which were entirely asporogenic. The two different types of cells possessed different morphological and physiological characteristics. The sporogenic cells were elongated, liquefied gelatin and stained blue with iodine. The asporogenic cells were more oval, liquefied gelatin less readily and stained yellow with iodine. According to Guilliermond (1919), Beijerinck secured similar results with *Schizosaccharomyces pombe* when cultivated on nutrient gelatin. He obtained a white colony composed of sporogenic cells and a brown colony having asporogenic cells.

It has also been noted that loss of sporulation may be accompanied by a loss of sexuality. This has been observed with *Schizosaccharomyces mellacei* and *Saccharomyces Ludwigii*.

Saito, according to Guilliermond (1919), observed two types of colonies in *Zygosaccharomyces mandshuricus*. One type was a transparent yellow colony containing asporogenic cells with a small amount of glycogen, the cells of which were long and sometimes formed chains. They liquefied gelatin. The other type of colony was white. Most of the cells of this type of colony were spherical, contained a large amount of glycogen, produced ascospores and did not liquefy gelatin.

It is evident from this brief review of the literature that many investigators have been able to induce or have observed morphological and colonial changes in different species of yeasts. When one turns to physiological changes, the results are less striking. Hansen working with *Saccharomyces turbidans*, a bottom yeast, was able to transform it into a top yeast by keeping it at a temperature of 5°C. On the other hand, he was not able to transform *Saccharomyces validus*, a typical top yeast, into a bottom yeast. In general he found that bottom yeasts could be converted into top yeasts but found that it was much more difficult to convert top yeasts into bottom yeasts. In the fermentation reactions, he was never able to suppress alcoholic fermentation entirely but was able to increase or decrease it.

Hansen was of the opinion that the composition of the medium was not an important factor in inducing variations in yeasts. He did not believe that the addition of materials such as various salts, peptone, or maltose to a solution or the use of must gelatin made any difference in inducing transformation. He was of the opinion that aeration did not make any difference in this respect. The only factor which seemed to have any effect was temperature. He was able to induce all his variations by extremes of temperature abnormal to the yeasts.

Present work

In trying to revive a dried-up agar slant culture of *Saccharomyces cerevisiae* Hansen, Saaz strain,² a culture of diplococci was obtained. Upon serial transfer in malt extract broth, this diplococcic form was gradually changed back into the original form. The present work was undertaken to determine whether this was a dissociant of the yeast, comparable to dissociated forms in bacteria, or whether the production of this form was purely accidental and if so whether it was possible to reproduce it. It raised the question also whether all yeasts do not under certain conditions undergo morphological, cultural and physiological changes.

Description of cultures used

The cultures used were: *Saccharomyces cerevisiae* Saaz, an industrial yeast, producing a bottom alcoholic fermentation. The cells are spherical and produce no scum in beer wort. The temperature limits for budding in beer wort are 3° to 40°C. Ascospore formation occurs with the production of from two to four ascospores. Agar slant cultures have a smooth, white glistening appearance. For morphological, cultural and physiological characteristics of this yeast see tables 1 and 2 and figures 1, 4, 7 and 10.

Saccharomyces cerevisiae Froberg, a yeast having morphological

² Hereafter for the sake of brevity this will be referred to as *Saccharomyces cerevisiae* Saaz while *Saccharomyces cerevisiae* Hansen, the Froberg strain, will be referred to as *Saccharomyces cerevisiae* Froberg.

and cultural characteristics similar to those of *Saccharomyces cerevisiae* Saaz, but producing a slightly less active alcoholic fermentation (see tables 1 and 2).

Saccharomyces ellipsoideus Hansen, a yeast producing a bottom fermentation. In beer wort cultures it produces either round or

TABLE 1
Showing the fermentation reactions of the various forms of yeasts

CULTURE	FORM OF YEAST	ARABINOSE	GLUCOSE	LEVULOSE	GALACTOSE	SUCROSE	MALTOSE	LACTOSE	RAFFINOSE	GLYCEROL	MANNITOL	DEXTRIN
<i>S. cerevisiae</i> Saaz..	S*	-	0	0	0	0	0	-	0	-	-	-
	R*	-	0	0	0	0	0	-	0	-	-	-
	G*	-	3+	2+	2+	3+	4+	3+	-	3+	+	+
<i>S. cerevisiae</i> Froberg	S	-	0	0	0	0	0	-	0	-	-	-
	R	-	0	0	0	0	0	-	0	-	-	-
	G	-	2+	2+	-	4+	-	-	-	-	+	-
<i>S. ellipsoideus</i> ..	S	-	0	-	-	0	0	-	-	-	-	-
	R	-	0	-	-	0	0	-	-	-	-	-
	G	-	3+	+	-	4+	-	-	-	2+	+	-
<i>Willia anomala</i>	S	-	0	0	-	0	-	-	0	⊕	-	-
	R	-	0	0	-	0	-	-	0	⊕	-	-
	G	-	2+	2+	-	4+	3+	-	-	2+	-	-
<i>Z. mandshuricus</i> ..	S	-	0	0	-	0	-	-	-	⊕	-	-
	R	-	0	0	-	0	-	-	-	⊕	-	-
	G	-	+	4+	-	-	-	-	+	+	-	-

0 = alcohol and CO₂; ⊕ = acid and gas; + = acid, figures indicate intensity of reaction; - = no action.

*S = smooth form; R = rough form; and G = gonidial form.

elliptical cells. The temperature limits for budding in beer wort are from 0.5° to 40°C. Scum formation occurs on liquid media. The cells in the scum may be greatly elongated. Ascospore formation occurs; the asci are ordinarily small and ellipsoidal and enclose from one to four ascospores. In about half of the cases, they germinate after having conjugated two by two (Marchaud).

Agar slant cultures appear dull, white, and usually slightly wrinkled. For detailed morphological and physiological characteristics see tables 1 and 2 and figure 11.

Willia anomala Saito, a spherical yeast producing a white wrinkled scum on liquid media. Agar slant cultures appear dull, white, and pebbly or wrinkled. Sporulation occurs with the production of from one to four ascospores per ascus. The ascospores are shaped like a hat with a projecting edge. An

TABLE 2

Showing size of cells, gelatin liquefaction, alcohol production, and sporulation of the different forms of the yeasts studied

CULTURE	SIZE OF CELLS IN MICRONS			GELATIN LIQUEFACTION AT END OF FOUR WEEKS			ALCOHOL PRODUCTION AFTER FOUR WEEKS IN CIDER			SIZE OF ASCOSPOR IN MICRONS			
	S	R	G	S	R	G	S	R	G	S	R		
							per cent	per cent					
<i>S. cerevisiae</i> Saaz.....	6	4x14-20	1	-	-	-	5	3	4	8	-	3.5-4	3.5-4
<i>S. cerevisiae</i> Froberg.....	6.5	3x14	1x1.5	-	-	+	4.2	4	0	-	-	3.2-3.5	3.4-5
<i>S. ellipsoideus</i> ...	5x8	2-6x16	1x1.5	-	-	+	6.2	4	7	-	-	2.5x3	5.2-8.4
<i>W. anomala</i>	5	6-8x14-20	1-1.5	-	-	+	4.2	2	8	-	-	3-4	2-2.5
<i>Z. mandshuricus</i>	6.5-9.5	10-20	1.2x1.5	-	-	+	3.4	3	2	-	-	3.5x4	5.3-5.4

+ = present; — = absent.

S = smooth form; R = rough form; G = gonidial form.

alcoholic fermentation is produced. Upon carbohydrate media a fruity odor is given off due to ester formation. For detailed morphological and physiological characteristics see tables 1 and 2.

Zygosaccharomyces mandshuricus Saito. Saito isolated this yeast from Chinese yeast used in making an alcoholic drink from Sorgho. The cells are round or oval. Agar slant cultures are white, smooth, and glistening. Asci are formed containing from one to four ascospores. These result from an isogamic conjugation. This yeast produces an alcoholic fermentation. For detailed morphological and physiological characteristics see tables 1 and 2.

Methods

All the cultures were serially transferred in malt-extract broth until upon plating all colonies presented an entire edge under low power magnification. The cultures were then single-celled, using Chambers' modification of the Barber technique. After a single cell isolation had been obtained of each of the yeasts, the following methods, well known in the study of bacterial dissociation, were used to obtain the different forms of yeasts.

(a) *Aging, and serial transfer in lithium chloride broth.* The lithium chloride broth was made by the addition of 0.25 per cent LiCl to nutrient broth and adjusting to pH 7. Rapidly growing cultures of the yeasts were used. These were plated out before use to verify smooth colonial appearance. Inoculations were then made into lithium chloride broth and serial transfers into new lithium chloride broth were made every two days until growth failed to appear in the last inoculated tube. Platings were made from the lithium chloride broth every two days after each inoculation using both glucose agar and malt extract agar. Colonies showing variations from the normal were selected from the plates and transfers made to agar slants. Simultaneously, hanging drop preparations were made of the lithium chloride cultures and morphological studies conducted to observe the changes occurring in this medium. As a control upon lithium chloride broth, cultures of the yeasts were inoculated into malt extract broth at the same time and platings made from this at the same time as from the lithium chloride broth. Cultures of the yeasts were also allowed to age in both the lithium chloride broth and in the malt extract broth. Platings were made every two days from each of these broths to observe the influence of aging in these broths upon the yeasts.

(b) *Aging, and serial transfer in brilliant green medium.* The effect of brilliant green upon the yeasts was determined by using a medium of the following composition: 10 grams peptone (Witte); 20 grams meat extract; 7 cc. of 1 per cent brilliant green; 7 cc. of a saturated solution of picric acid; 1000 cc. of distilled water.

The yeasts were transferred serially every two days in the brilliant-green medium and plated after forty-eight hours incuba-

tion in this medium. They were also allowed to age in this medium and platings made every two days from the tubes which had aged for different lengths of time.

(c) *Influence of high concentration of alcohol on yeasts.* Ethyl alcohol was sterilized by refluxing in a sterile condenser for one hour. This sterile alcohol was then added to malt extract broth to make broths containing 5, 10, 15, 20, 25, 30, 40 and 50 per cent alcohol (see table 4). Platings and hanging-drop preparations were made of the yeasts at the end of the first and second week after inoculation in the different concentrations of alcohol. Control tubes consisted of uninoculated tubes of the various con-

TABLE 3

Showing influence of aging in lithium chloride on the dissociation of the S form of the different yeasts

CULTURE	FIRST APPEARANCE	
	R	G
	days	days
<i>S. cerevisiae</i> Saaz	12	6
<i>S. cerevisiae</i> Froberg	14	22
<i>S. ellipsoideus</i>	6	12
<i>W. anomala</i>	4	10
<i>Z. mandshuricus</i>	6	14

W. anomala, naturally rough, required three weeks rapid transfer in malt broth to get smooth.

centrations of alcohol which were examined for sterility, and also cultures in alcohol-free malt extract broth. These were examined at the same time and in the same way as the cultures in the alcohol media.

(d) *Effect of desiccation on the yeasts.* Actively-growing malt extract broth cultures of the yeasts were seeded on sterile gypsum blocks and allowed to desiccate. At the end of one week hanging drop preparations and plate cultures were made from the blocks.

(e) *Effect of temperature on the yeasts.* Plain broth and malt extract broth cultures of the yeasts were incubated at temperatures of 9°, 17°, 23°, 29° and 37°C. (see table 5). Hanging-drop preparations were examined and agar-plate cultures made

from the two different broths at weekly intervals for a period of one month.

Definition of terms

Certain definite colonial, morphological, cultural and physiological forms have occurred constantly in the yeasts when they

TABLE 4

Showing influence of alcohol on dissociation of the S form of the yeasts

CULTURE	PERCENTAGE OF ALCOHOL							
	5	10	15	20	25	30	40	50
At end of one week incubation at 20°C								
<i>S. cerevisiae</i> Froberg..	per cent 5 G	per cent 10 G	per cent 20 G	per cent 50 G	per cent 100 G	per cent Few cells all G	per cent Very few cells all G	per cent No cells pres- ent
<i>S. cerevisiae</i> Saaz	No G	No G	5 G	25 G	30 G	Few cells all G	Very few cells all G	No cells pres- ent
<i>S. ellipsoideus</i>	10 S 85 R 5 G	50 R 50 G	100 G	100 G	100 G	Few cells all G	No cells pres- ent	No cells pres- ent
Two weeks incubation at 20°C.								
<i>S. cerevisiae</i> Froberg.	100 S	100 G	100 G	No cells pres- ent	No cells pres- ent	No cells pres- ent	No cells pres- ent	No cells pres- ent
<i>S. cerevisiae</i> .	100 S	100 S	50 S 50 G	100 G	No cells pres- ent	No cells pres- ent	No cells pres- ent	No cells pres- ent
<i>S. ellipsoideus</i>	10 S 90 R	50 R 50 G	10 R 90 G	No cells pres- ent	No cells pres- ent	No cells pres- ent	No cells pres- ent	No cells pres- ent

S = smooth form of yeast; R = rough form of yeast; G = gonidial form.

Per cent refers to the number of cells of each form observed based on the total number of cells present.

were grown under the above described conditions. For the purpose of clarity the salient characteristics of these different forms will be briefly described.

The smooth form of a yeast is considered as one having uniform and regular morphology, which, when plated on agar, forms a

TABLE 5

Showing effect in malt extract broth and nutrient broth of temperature on dissociation in yeast

CULTURE	ONE WEEK			TWO WEEKS			THREE WEEKS			FOUR WEEKS		
	S	R	G	S	R	G	S	R	G	S	R	G
37°C.												
<i>S. cerevisiae</i> Saaz	+	-	-	+	+	-	+	+	+	-	-	+
<i>S. cerevisiae</i> Froberg . .	+	-	-	+	+	-	+	+	-	+	+	-
<i>S. ellipsoideus</i>	+	-	-	-	+	-	-	+	+	-	+	+
<i>W. anomala</i>	+	+	-	+	+	-	-	+	-	-	+	+
<i>Z. mandshuricus</i>	+	-	-	+	-	-	+	+	-	+	+	+
29°C.												
<i>S. cerevisiae</i> Saaz	+	-	-	+	+	-	+	+	-	+	+	+
<i>S. cerevisiae</i> Froberg . . .	+	-	-	+	-	-	+	-	-	+	-	-
<i>S. ellipsoideus</i>	+	-	-	+	+	-	+	+	-	+	+	-
<i>W. anomala</i>	+	-	-	+	+	-	+	+	-	+	+	-
<i>Z. mandshuricus</i>	+	-	-	+	-	-	+	-	-	+	+	-
23°C.												
<i>S. cerevisiae</i> Saaz	+	-	-	+	-	-	+	-	-	+	-	-
<i>S. cerevisiae</i> Froberg . . .	+	-	-	+	-	-	+	-	-	+	-	-
<i>S. ellipsoideus</i>	+	-	-	+	-	-	+	-	-	+	-	-
<i>W. anomala</i>	+	-	-	+	-	-	+	-	-	+	-	-
<i>Z. mandshuricus</i>	+	-	-	+	-	-	+	-	-	+	-	-
17°C.												
<i>S. cerevisiae</i> Saaz	+	-	-	+	-	-	+	-	-	+	-	-
<i>S. cerevisiae</i> Froberg . . .	+	-	-	+	-	-	+	-	-	+	-	-
<i>S. ellipsoideus</i>	+	-	-	+	-	-	+	-	-	+	-	-
<i>W. anomala</i>	+	-	-	+	-	-	+	-	-	+	-	-
<i>Z. mandshuricus</i>	+	-	-	+	-	-	+	-	-	+	-	-
9°C.												
<i>S. cerevisiae</i> Saaz	+	-	-	+	-	-	+	-	-	+	-	-
<i>S. cerevisiae</i> Froberg . . .	+	-	-	+	-	-	+	-	-	+	-	-
<i>S. ellipsoideus</i>	+	-	-	+	-	-	+	-	-	+	-	-
<i>W. anomala</i>	+	-	-	+	-	-	+	-	-	+	-	-
<i>Z. mandshuricus</i>	+	-	-	+	-	-	+	-	-	+	-	-

+ = present; - = absent.

S = smooth form; R = rough form; G = gonidial.

smooth glistening colony with an entire edge under low power magnification. Agar slant cultures appear smooth and glistening. The physiological reactions are the same as those commonly ascribed to the species. The smooth form of the yeast is usually the normal form by which the yeast is commonly recognized, although there are exceptions to this as will be noted later. The smooth form of yeasts will be designated hereafter as the "S" form (see tables 1 and 2 and figures 1, 4, 7, 10 and 11).

The rough form of a yeast is considered as one having slender, greatly elongated or irregular shaped cells. The colonial forms are rugose, dull and when viewed under low power magnification, they have an irregular filamentous edge resembling mycelial growth in the molds. Agar slant cultures are likewise dull and rugose, often having a powdery appearance. The rough form will be referred to hereafter as the "R" form (see tables 1 and 2 and figures 2, 5, 8, 10 and 12).

A third form of yeast which has been produced regularly is what we have termed the gonidial form which will be designated hereafter as the "G" form. The term "G" form as defined by Hadley *et al.* (1931) and commonly used by many bacteriologists when working with bacteria has reference to the colonies, microscopic in size, produced by coccoid forms which were filterable through various types of filters. The "G" form referred to throughout this paper has reference not to the colonial forms, but to the gonidial form which is produced during the dissociation of the yeast. The colony produced by the "G" form is a large thin spreading colony many times larger than the S or R colonies in most cases. The G form of a yeast consists of either spherical or rod-shaped cells greatly reduced in size from that of the normal yeast. Colonies on agar plates usually grow slowly at first, in many cases being microscopic in size after a week's incubation. Under low power magnification they may present either an entire edge or an irregular edge. In the later case the colonies resemble bacterial roughs. However, in the majority of yeasts studied so far, after the G forms have become adapted to growing on culture media, they grow very rapidly and produce a large, flat, adherent, spreading colony. (See figures 6, 9 and 13.) Not only are the

morphological and cultural characters of the G form of the yeast widely different from those of the S and R forms of the same yeast but also its physiological characters, since it no longer produces alcoholic fermentation with gas, but an acid fermentation without gas (see tables 1 and 2).

A fourth form of yeast cell has also been observed constantly in this work. This form has been designated as the transitional form and will be referred to hereafter as the "T" form. The "T" form has never been cultured and has been observed only under the microscope. This form of the yeast may be either oval or elongated depending upon the particular yeast studied. They are practically the same size as the cells of the culture in which they appear. They are seen in both S and R cultures and are characterized by being more highly refractive than the other cells and not readily stained. If such cells are isolated and observed carefully for several hours in a moist chamber, a number of small budding forms or "gonidia" appear on the periphery of the cell. These small forms are the gonidial forms which have just been described as the G form of the yeast. These T forms have been single-celled a great many times and they have always produced the G forms and never their own kind. They are formed in all the media and under all the physical conditions used in this work. They were produced most abundantly in the malt extract broth containing different percentages of alcohol. They were always observed when the S and R forms of the yeast were being dissociated into the G form but were never observed when the G form of the yeast was reverting to the R and S forms.

RESULTS

Description of the various forms obtained

As previously stated, all the cultures used in this study were first reduced to what was considered the smooth or S form. After they had been reduced to this form, they were isolated by Chambers' modification of the Barber single cell technique. The cultures obtained from a single cell were then serially transferred into the various media, viz., lithium chloride broth, brilliant green

broth and malt extract broth containing 5, 10, 15, 20, 25, 30, 40 and 50 per cent alcohol respectively. They were also grown in plain and malt extract broths at temperatures of 9°, 17°, 23°, 29° and 37°C. and subjected to desiccation on plaster of Paris blocks. Microscopic examinations of the cultures were made every other day to determine the appearance of the different forms. The cultures were also plated on malt extract and glucose agar every other day to check the colonial forms with the morphological observations. It was necessary to use both malt extract and glucose agar since the G forms did not grow readily on malt extract agar from the primary isolation. In fact it required at least five days for them to grow out on glucose agar. However, once colonial forms had been obtained, subsequent transfers grew readily on either malt extract or glucose agar.

Since the different yeasts reacted differently to various conditions and each presented different morphological changes, it will be necessary to consider them separately.

INDUCED FORMS OF *SACCHAROMYCES CEREVISIAE* SAAZ

In lithium chloride broth the G forms of *Saccharomyces cerevisiae* SaaZ began to appear in the microscopic preparations after six days aging or after being serially transferred every other day for three times. However, they were completely overgrown on the plates by the S forms. The R forms did not appear in the microscopic preparations or on the plates until the cultures had been aged in lithium chloride broth for 12 days or had been serially transferred for the same length of time. At the end of this period elongated cells averaging 14 to 20 microns in length, began to appear. The R cells produced rugose, dull, wrinkled colonies with filamentous projections extending from 1 to 2 cm. from the colonial mass. Single-cell isolations of this R form have remained stable on culture media for ten months (see tables 1 and 2 and figures 2, 5, and 8).

After several rapid transfers of the R forms in lithium chloride broth, or after aging in this medium for two weeks or longer, G forms were obtained. It was, therefore, possible to obtain G forms of the yeast both from the S form and the R form of the

yeast by several rapid transfers in lithium chloride broth or by aging in this medium for some time. It required the same length of time for the G forms to appear by either method. The advantage of serial transfers over the aging method was that in the former method the S or R form was gradually eliminated or greatly reduced in number. For example, if cultures of the S form were used, by serial transfer the number of S forms gradually diminished and there was a corresponding increase of R and G forms.

The G forms obtained in the case of *Saccharomyces cerevisiae* Saaz were small diplococci, one micron in diameter, exactly like the culture first encountered from the dried tube of agar which had been kept eighteen months. The colonies were entire and remained microscopic in size even after five days incubation. However, after this time they apparently became adjusted to the medium and formed thin, dull pebbly macroscopic colonies. The pebbly appearance of the colonies was due to the formation of secondary colonies. Upon aging, the colony turned to a pale yellow color. (See tables 1 and 2 and figures 3, 6, 9 and 10.)

The control tubes, consisting of cultures of the yeast undergoing aging, when serially transferred in malt extract broth yielded only the S form of the yeast. No changes occurred at any time.

When the S form of *Saccharomyces cerevisiae* Saaz was serially transferred in brilliant green broth, R forms appeared within 24 hours. The S form of the culture was completely changed to the R form in this time. The morphological and cultural aspects of the R form corresponded to those obtained in lithium chloride broth which have been previously described. In this medium G forms appeared at the end of four days aging or after the second transfer. After the third transfer in this medium subsequent transfers failed to grow and at the end of a week, it was impossible to obtain growth from any of the tubes. When the concentration of the brilliant green was reduced to a point which permitted the yeast to grow indefinitely, no variation occurred. Under these conditions only the S form of the yeast appeared. The G forms obtained in this medium were identical in all respects with those obtained in lithium chloride broth. Cultures of the yeast

in malt extract broth, run as a control, remained in the stable S form, showing no variations.

In malt extract broth containing different percentages of alcohol no R forms of *Saccharomyces cerevisiae* Saaz were obtained. At the end of one week's incubation at 20°C., G forms of the yeast began to appear. Agar plates made from the tubes showed that the number of G forms appearing depended on the amount of alcohol present, up to a certain percentage. After this the concentration of the alcohol was germicidal to the yeasts. These data are given in tabular form in table 4. The G forms were identical in all respects with those obtained in the lithium chloride and brilliant green broths. Control cultures of the yeast in alcohol-free malt extract broth remained in the stable S form.

When the S forms of *Saccharomyces cerevisiae* Saaz were placed in malt extract and nutrient broth and incubated at temperatures of 9°, 17°, 23°, 29° and 37°C., various changes in the yeast form occurred. At the higher temperatures, 29° and 37°C., the R forms began to appear at the end of two weeks. At the end of four weeks at 37°C. both the S and R forms had disappeared and only the G form was present. At 29°C. at the end of four weeks all three forms were present. At the lower temperatures the S form remained stable and there was no evidence of either the R or G forms (see table 5).

On plaster of Paris blocks *Saccharomyces cerevisiae* Saaz, when examined at the end of a week showed no R forms but both S and G forms were obtained.

With single cell isolations from a typical culture of *Saccharomyces cerevisiae* Saaz, which was considered as the S form of the culture, it was possible to convert the strain into two other distinct forms by serially transferring it in lithium chloride broth, or brilliant green broth or by allowing it to age in these media, or by aging in different percentages of alcohol. Similar results were obtained by aging at temperatures of 29° and 37°C. or by desiccation on gypsum blocks. The morphological and colonial appearance of the R cultures was quite different from that of the S cultures (see tables 1 and 2 and figures 1 to 10). The fermentation reactions of the R form were the same as those of the S form.

However, the G forms produced were radically different from those of either the S or R forms. They were much smaller, different in shape, produced a different type of colony and possessed entirely different fermentation reactions. The S and R forms fermented the same sugars with the production of alcohol and carbon dioxide, while the G forms produced no alcoholic fermentation but an acid fermentation. In addition to causing an acid instead of an alcoholic fermentation, the G form fermented all the sugars that the S and R forms fermented, except raffinose, and in addition caused an acid fermentation in lactose, glycerol, mannitol and dextrin (see table 1). The G form of this yeast was asporogenic, while both the S and R forms were sporogenic (see table 2). Thermal-death-point determinations on the S, R and G forms of *Saccharomyces cerevisiae* Saaz revealed that the G form was more resistant to heat than either the S or R form. The G form had a thermal death point of 56°C., while the S and R forms run at the same time and under the same conditions had a thermal death point of 52°C.

Reversion of G and R forms to S forms

After the G forms had been obtained, they were single-celled and two different cultures of single-cell isolations were serially transferred in malt extract broth every other day for a period of two weeks. At the end of this time both of the cultures from the single cell isolation gave tetrad forms, the diameter of which was 3 to 3.5 microns. Upon transferring the tetrad forms for two weeks longer in malt extract broth, one of the cultures gave three different colonial forms. One of the colonial forms obtained was an S form which produced smooth pink colonies. The cells were spherical and were from 5 to 6 microns in diameter. When these pink colonies were transferred to a glucose or malt agar slant, they produced an abundant pink growth which after four or five days became mucoid in character and ran off the slant to the bottom of the tube where it collected in a large viscous mass. Subsequent transfers on agar slants behaved similarly. This mucoid form was serially transferred for three weeks in malt extract broth at the end of which time, it had completely reverted

to the normal S form. Another colonial form obtained from this culture was a white wrinkled powdery R form similar to those obtained from lithium chloride broth and from the other methods used in converting the S forms to the R forms. The cells were 4 by 12 microns. After sixteen days serial transferring of this R form, it reverted to the normal S form. The third colonial form obtained from this single cell culture was an R form which produced dull black wrinkled colonies. The size of the cells of the black form was 4 by 12 microns. After transferring serially for twenty-two days in malt extract broth, they reverted to normal S forms.

The other culture obtained from a single cell isolation was transferred at the same time and under the same conditions in malt extract broth for a period of six weeks. At the end of this time it reverted to the normal S type without producing the pink S form or the white R form or black R form. However, as previously stated, it produced the tetrad forms at the end of two weeks the same as the other single cell culture.

SUMMARY

In the case of *Saccharomyces cerevisiae* Saaz, it was possible to convert single cell isolations of the normal S form by means of chemicals added to suitable media and by physical influences into typical R and G forms having certain morphological, cultural and physiological characteristics. In the case of the R form, cultures were obtained which have remained stable for a period of ten months and in the case of the G forms cultures were obtained which have remained stable for a period of fourteen months.

It has likewise been possible to make single-cell isolations of the R and G forms and by serially transferring these single-cell cultures in malt extract broth to change them back into the normal S form. The reversion process may take place in a direct manner or several intermediates may be formed such as pink mucoid forms or black rough forms, both of which, upon continued transfer, revert to the normal S form.

Induced forms of Saccharomyces cerevisiae Froberg

In lithium chloride broth the R forms of *Saccharomyces cerevisiae* Froberg began to appear in the microscopic preparations and on the plates made from the lithium chloride broth after fourteen days aging in the broth, or after being serially transferred in the broth for the same period of time. The R cells were long slender rods, 3 by 14 microns in size. Macroscopically they produced dull, rugose, wrinkled colonies the edges of which under low magnification were filamentous and extended about one centimeter from the colonial mass. Single cell isolations of this R form reverted to the normal S form at the end of three weeks aging on agar. No stable R form was obtained.

After continued aging or serial transfer of the S form in lithium chloride broth for a period of twenty-two days G forms were obtained. G forms were also obtained by aging or serial transfer of the R form in lithium chloride broth for a period of two weeks.

The G forms obtained from *Saccharomyces cerevisiae* Froberg were small rods which measured 1 by 1.5 microns. At the end of three days on agar plates, the colonies were macroscopic in size and had an irregular edge, the colony resembling the colonies formed by R forms of bacteria. After five days of incubation at room temperature on an agar plate, the G form gave a thin, dull gray, adherent, spreading colony. Upon subsequent transfer to sterile agar plates, colonies were produced which spread over the entire surface of the plate in one week's time. Cultures of the S form in malt extract broth, run under the same conditions and in the same manner as the lithium chloride tubes, remained stable.

When the S form of *Saccharomyces cerevisiae* Froberg was aged in or serially transferred in brilliant green broth, R forms appeared within twenty-four hours. The morphological and cultural aspects of the R form corresponded to those obtained from lithium chloride broth. Single cell isolations of this R form likewise reverted to the normal S form at the end of three weeks on agar slants exactly as had the R forms obtained from lithium chloride broth. In brilliant green broth G forms appeared after six days aging or after three serial transfers. After the fourth

transfer in this medium, subsequent transfers failed to grow and at the end of ten days it was impossible to obtain growth from any of the tubes. The G forms obtained in this medium were identical with those obtained in lithium chloride broth; control tubes remained in the stable S form.

In malt extract broth containing different percentages of alcohol, no R forms of *Saccharomyces cerevisiae* Froberg were obtained. However, G forms appeared at the end of one week's incubation at 20°C. The percentages of S and G colonies developing on agar plates made from the tubes revealed that the number of G forms present was directly dependent on the concentration of the alcohol in the media (see table 4). The G forms obtained in these alcohol media were identical with those obtained in the lithium chloride and brilliant green broths. Control cultures in alcohol-free malt extract broth remained stable in the S form.

When the S form of *Saccharomyces cerevisiae* Froberg was inoculated in malt extract and nutrient broth and incubated at temperatures of 9°, 17°, 23°, 29° and 37°C., R forms were obtained only at a temperature of 37°C. At this temperature, R forms began to appear at the end of two weeks and at the end of four weeks both S and R forms were present in about the same proportion. No G forms were obtained at any time. At the lower temperatures the S form remained stable with no appearance of either R or G forms in the tubes.

On plaster of Paris blocks *Saccharomyces cerevisiae* Froberg produced no R forms. When examined at the end of one week, both S and G forms were obtained.

By the methods outlined above, a typical culture of *Saccharomyces cerevisiae* Froberg obtained from a single cell isolation considered as the S form was converted into two other distinct forms, the R and G forms. The morphological and cultural characteristics of the R form were very different from those of the S form. However the R form possessed the same fermentation reactions as the S form (see table 1). The G forms obtained differed greatly from either the S or R forms. They were much smaller, different in shape, being short rods, had a different colo-

nial formation and entirely different fermentation reactions. Whereas the S and R forms produced an alcoholic fermentation with gas, the G forms produced an acid fermentation. The G form had lost the ability to ferment maltose, galactose, and raffinose but had retained the ability to ferment sucrose, glucose and levulose. The S and R forms fermented all of these sugars. In addition to this the G form gave an acid fermentation in mannitol, while the S and R forms did not ferment mannitol. The G form of this yeast also differed from the S and R form in that it was asporogenic and liquefied gelatin. The S and R forms were both sporogenic and did not liquefy gelatin at the end of four weeks (see table 2).

Reversion of R and G forms to S forms

Typical cultures of the R and G forms were obtained in single-cell isolations. The resulting R and G cultures from these single-cell isolations were planted in malt extract broth and serially transferred every other day. At the end of ten days the R form had completely reverted to the normal S form. Cultures of the R form on agar slants also reverted to the S form at the end of three weeks. After being serially transferred in malt extract broth for twenty-four days, the G form reverted to the normal S form. This reversion was abrupt, no intermediate forms appearing.

*Induced forms of *Saccharomyces ellipsoideus**

In lithium chloride broth R forms of *Saccharomyces ellipsoideus* began to appear after six days aging of the S form in this broth, or after serial transfer of the S form every other day for a period of six days. The R cells were long slender rods from two to six microns in diameter and averaging 16 microns in length. They produced dull, rugose, wrinkled colonies the edges of which when examined under lower-power magnification showed very fine, slender filamentous chains of cells extending from three to five centimeters from the colonial mass (fig. 12). Single cell isolations of this R form have remained stable on agar slants for five months.

After further aging or serial transfer of the R form in lithium

chloride broth, G forms appeared at the end of twelve days. The G forms obtained from *Saccharomyces ellipsoideus* were small rods of 1 by 1.5 microns. On agar plates, the colonies remained microscopic in size growing very slowly for the first five days. However, after this time they developed more rapidly and after two weeks formed a dull gray, thin, adherent, spreading colony, 5 to 7 cm. in diameter, with an irregular edge (fig. 13). Agar slant cultures had a thin spreading growth and produced a dark greenish discoloration of the medium. S cultures in malt extract broth, run under the same conditions and in the same manner as the lithium chloride cultures remained stable and contained nothing but S cells.

When the S form of *Saccharomyces ellipsoideus* was aged or serially transferred in brilliant green broth, R forms were obtained at the end of two days. Morphological and cultural aspects of this R form were identical with those obtained from lithium chloride broth. In the brilliant green broth G forms appeared at the end of four days aging or after the second serial transfer. After the fourth serial transfer, subsequent transfers failed to grow and at the end of eight days it was impossible to obtain growth from any of the tubes. This G form was similar in every detail to the G forms obtained from lithium chloride broth. Control cultures in malt extract broth remained stable in the S form. No other forms were obtained from the tubes.

In malt extract broth containing different percentages of alcohol no R forms of *Saccharomyces ellipsoideus* were obtained. At the end of one week's incubation at 20°C., G forms began to appear in certain tubes (see table 4) depending upon the concentration of alcohol. The G forms obtained were similar to those in the other two media. Control cultures in alcohol-free malt extract broth did not produce the G form, but were stable and remained in the S form.

When the S form of *Saccharomyces ellipsoideus* was planted into malt extract and nutrient broth and incubated at temperatures of 9°, 17°, 23°, 29° and 37°C., changes occurred in the form of the yeast only at the two higher temperatures. At the end of two weeks at 37°C., only the R form of the yeast was

present. At the end of the third week G forms began to appear and at the end of the fourth week were present in large numbers. At 29°C., R forms began to appear at the end of two weeks and at the end of four weeks both S and R forms were present. At this temperature the S forms were more stable than at 37°C. since they persisted for a much longer time. No G forms occurred at this temperature. At the lower temperatures, the S form remained stable; there was no appearance of either the R or G forms (see table 5).

On plaster of Paris blocks *Saccharomyces ellipsoideus* produced no R forms. G forms were obtained upon examination of the blocks at the end of one week.

By the methods outlined above, a typical culture of *Saccharomyces ellipsoideus*, from a single cell isolation, considered as the S form, gave rise to two other distinct forms, the R and G forms. The R form differed from the S form in morphological and cultural characteristics but possessed the same fermentation reactions. The G forms differed greatly from either the S or R form. They were much smaller, being short rods, had a different colonial formation, and different fermentation reactions. Whereas, the S and R forms produced an alcoholic fermentation with gas, the G form produced an acid fermentation. The G form fermented the same sugars as the S and R forms with the exception of maltose, and in addition caused an acid fermentation in mannitol, glycerol, and levulose (see table 1). The G form of this yeast liquefied gelatin at the end of one week, while the S and R forms did not liquefy gelatin. The G form of this yeast differed from the S and R forms in another respect. Both the S and R forms were sporogenic; the G form was asporogenic (see table 2).

Reversion of the R and G forms to the S form

Typical stable cultures of the R and G forms were obtained in single cell isolations. The resulting R and G cultures from these isolations were planted in malt extract broth and serially transferred every two days. At the end of twenty-eight days the R form had completely reverted to the S form. In the case of the G form, reversion to the S form took place after thirty-four days

of serial transfer in malt extract broth. This reversion of the G to the S form was abrupt, no intermediate forms appearing.

Induced forms of Willia anomala

In the case of *Willia anomala* the culture that we had at the start was an R form. After three weeks serial transfer in malt extract broth, this R form gave smooth typical S form colonies. This S form was single-celled and has since remained as a stable S form.

When the S form of *Willia anomala* was planted into lithium chloride broth and allowed to age, or was serially transferred in the broth, R forms appeared at the end of four days. No S forms were present in the cultures, the conversion to the R form being complete in this time. The R cells were from 6 to 8 by 14 to 20 microns in size. The R cells produced white, rugose, wrinkled, powdery colonies the edges of which were filamentous. Under low power magnification the filaments were visible as long chains of the R forms. The filaments were relatively large and extended 1 cm. from the colonial mass. Single-cell isolations of this R form have remained stable on agar slants for nine months.

After continued aging or serial transfer of this R form in lithium chloride broth, at the end of ten days G forms appeared. The G forms obtained from *Willia anomala* were small diplococci measuring from 1 to 1.5 microns in diameter. The colonies on agar plates grew very slowly, and were microscopic in size, for the first five days after plating. After this time they grew more rapidly, forming dull light gray, spreading, adherent colonies having an irregular edge. Upon agar slants, a darkening of the medium occurred.

The S form run as a control in malt extract broth remained stable and did not produce either the R or G form.

When the S form of *Willia anomala* was aged or serially transferred in brilliant green broth, 100 per cent conversion to the R form occurred in twenty-four hours. Upon further aging or serial transferring of the R form in this medium, G forms were obtained at the end of four days. After the fifth transfer subsequent transfers failed to grow and at the end of one week it was

impossible to obtain growth from any of the tubes. The R and G forms of *Willia anomala* obtained in this medium were identical with those obtained in lithium chloride broth. Control cultures in malt extract broth remained in the stable S form, showing no variation.

When the S form of *Willia anomala* was planted into malt extract and nutrient broth and these cultures incubated at temperatures of 9°, 17°, 23°, 29° and 37°C., changes in the form of the yeast occurred only at the two higher temperatures. At 37°C., R forms appeared at the end of the first week and at the end of the third week the culture was 100 per cent R. At the end of the fourth week G forms began to appear, both R and G forms being present. At 29°C., R forms appeared at the end of the second week and at the end of the fourth week both S and R forms were present. The presence of the S form at the end of four weeks in this case indicates the influence of temperature on dissociation since at 37°C. all the S forms had disappeared in a much shorter time. No G forms were obtained at this temperature. At the lower temperatures the S form remained stable; neither R nor G forms appeared in any of the tubes.

On plaster of Paris blocks *Willia anomala* produced no R forms. Upon examination of the blocks at the end of one week G forms were obtained.

By the methods outlined above, a culture of *Willia anomala*, from a single cell isolation, considered as the S form, gave rise to two other forms, the R and G forms. The R form differed culturally and morphologically from the S form but possessed the same fermentation reactions. The G forms differed radically from the S and R forms. They were small diplococci, had a different colonial formation and fermentation reactions. The S and R forms produced an alcoholic fermentation with gas. The G form produced an acid fermentation without gas. The G form fermented the same sugars as the S and R forms with the exception of raffinose, and in addition caused an acid fermentation in maltose. Moreover, the G form of this yeast liquefied gelatin rapidly, while the S and R forms did not liquefy gelatin. Furthermore, the G form of this yeast differed from the S and R forms in

that it was an asporogenic form, while both the S and R forms were sporogenic (see table 2).

Reversion of the R and G forms to the S form

Typical R and G forms were obtained in single cell isolations. The resulting cultures were planted into malt extract broth and serially transferred in this medium every other day. At the end of three weeks the R form had been converted back to the S form. In the case of the G form, after serial transfer in this medium for thirty-four days, the S form was obtained. This reversion was abrupt and no intermediate forms were obtained.

*Induced forms of *Zygosaccharomyces mandshuricus**

When the S form of *Zygosaccharomyces mandshuricus* was aged or serially transferred in lithium chloride broth R forms appeared in six days. The R cells were of many shapes averaging from 6 to 9 microns wide and 10 to 20 microns long. Many bizarre forms occurred. The R cells formed dull, wrinkled colonies with a frilly edge. Single cell isolations of this R form reverted to the normal S form on agar slants at the end of two or three weeks aging.

Upon further transfer of the R form of the culture in lithium chloride broth, G forms appeared at the end of fourteen days. The G forms obtained from *Zygosaccharomyces mandshuricus* were small rods from 1.2 to 1.5 microns in length. The colonies grew slowly at first on agar plates and remained microscopic in size for five days after plating. At the end of this time they became adapted to growth on glucose agar and produced colonies about one centimeter in diameter at the end of two weeks. The G form produced dull smooth colonies with an entire edge. The colonies were bright orange in color. Agar slants likewise were bright orange in color. This G form was very adherent to the agar. Control cultures in malt extract broth, run at the same time and under the same conditions as the lithium chloride cultures, yielded only the S form, no variations occurring at any time.

When the S form of *Zygosaccharomyces mandshuricus* was

aged or serially transferred in brilliant green broth, no R forms were obtained. G forms were obtained in this medium at the end of six days aging or by serial transfer. The G form obtained in this medium was identical with those obtained in lithium chloride broth (see table 4). Control cultures in malt extract broth showed no change; the S form remained stable in this medium.

When the S form of *Zygosaccharomyces mandshuricus* was planted into malt extract and nutrient broth and incubated at 9°, 17°, 23°, 29° and 37°C., changes occurred in the form of the yeast at the two higher temperatures. At 37°C., R forms were obtained at the end of three weeks incubation. At the end of four weeks all three forms, the S, R, and G forms, were present. At 29°C., R forms appeared at the end of the fourth week, but no G form of this yeast was produced at this temperature. At the lower temperatures the S form remained stable; neither the R nor G forms appeared (see table 5).

On plaster of Paris blocks *Zygosaccharomyces mandshuricus* produced no R forms. Upon examination of the blocks at the end of one week G forms were obtained.

By the methods outlined above, a culture of *Zygosaccharomyces mandshuricus*, from a single cell isolation, considered as the S form, gave rise to two other forms, the R and G forms. The R form differed morphologically and culturally from the S form but possessed the same fermentation reactions. The G form differed greatly from the S and R forms, being much smaller, and presented different cultural appearances. The S and R forms produced an alcoholic fermentation with gas. The G form had very feeble fermentative powers, produced no alcoholic fermentation, but a very slight acid fermentation. The G form fermented all the sugars that the S and R forms did with the exception of sucrose, and in addition produced an acid fermentation in raffinose. The S and R forms did not liquefy gelatin in four weeks while the G form completely liquefied gelatin inside of a week. Furthermore, the G form of this yeast was an asporogenic form, while the S and R forms both formed spores.

Reversion of the R and G forms to the S form

Typical R and G forms were obtained in single cell isolations. The resulting cultures were planted into malt extract broth and serially transferred every other day. At the end of two days the R form had completely reverted to the normal S form. In the case of the G form, after six days of serial transfer in malt extract broth the G form had reverted to the S form. The change was abrupt; no intermediate forms of the yeast occurred.

Mechanism of the changing of an S form to an R or G form

During the course of studying the various morphological, cultural and physiological changes which took place in the different yeasts as they were transferred serially or aged in the various media or subjected to the different physical forces, it appeared that the changes occurred in an orderly manner. Starting with the smooth form of the yeast with typical morphological, cultural and physiological characteristics, there was a progressive change to the R form. The cells gradually changed to an elongated form with all degrees of elongation intervening between the normal S form of cells and the stable R form of cells which has been described previously and the characteristics of which are given in tables 1 and 2. Likewise, intermediate colonial forms were obtained varying in degree of roughness between the normal S type and the stable R type of colony which has been described earlier in the paper.

In the reversion of the R form to the S form the reverse process occurred. The cells gradually lost their elongated character and the colonial forms likewise gradually lost their wrinkled, rugose appearance and their filamentous edge until a complete transformation to the S form had occurred.

In the transformation of an S or R form to a G form two methods of change occurred. During the process of transformation cells ranging in size from the normal S form or from the typical R form to the G form occurred in the medium. Apparently, there was a gradual diminution in the size of the cells until the stable G form was reached. This method of change from the S to the G form was very common in *Saccharomyces ellipsoideus*

when grown in lithium chloride broth, or when grown at a high temperature. This type of change was less prevalent in the other yeasts studied. It was also less prevalent in *Saccharomyces ellipsoideus* when this yeast was grown in any of the other media.

The second type of change from an S or R form to a G form was of an altogether different character. Normal-sized S or R cells became highly refractile and were transformed into another form of the yeast which has been described earlier in the paper as the transitional form and which has been designated as the "T" form. These highly refractile T cells did not reproduce by normal budding as did the S and R cells, but by an entirely different process. A multitude of minute buds appeared on the periphery of the cell. These minute buds upon becoming detached from the cell, corresponded to the G form of the yeast. Numerous single cell isolations of these T cells which were covered with minute buds always gave rise to pure cultures of the G form. This type of transformation of an S form to a G form was observed in all the yeasts studied in all the different media and under all the physical conditions employed. This type of change was prevalent in all the liquid media used, and was especially so in the alcohol media where this was the only type of transformation that occurred.

Apparently then, the transformation of an S or R form to a G form may be either a gradual process accomplished by a graded diminution in the size of the cells, or may be an abrupt process accomplished by the formation of an intermediate T cell.

In the reversion of a G form to an S form the transition was either gradual with the formation of various intermediates as was the case with the G form of *Saccharomyces cerevisiae* Saaz, as described earlier in the paper, or was abrupt, giving no intermediate forms, as was the case with the G forms of all the other yeast studied. The T form was never encountered during the reversion process.

Relationship between scum formation and the different forms

Scum formation has always been used as one of the criteria or characteristics for the identification of yeasts. In view of this fact a series of experiments was set up to study the relationship

between scum formation and the various forms of the different yeasts. For this study malt extract broth and nutrient broth media were used. Single-cell isolations of the three forms, S, R, and G of each of the five different yeasts were inoculated into each of these media and incubated at temperatures of 9°, 17°, 23°, and 29° and 37°C. for a period of three months. There was no further change at the end of four weeks.

The results of these studies are given in detail in table 6, and only a general discussion of the results will be given here. Scum formation occurred in all the R forms of all the yeasts studied regardless of the temperature. The S form only produced scum at the two higher temperatures. It was at these same two temperatures that our previous studies showed dissociation of the S form to occur. Plates made from the S cultures when scum formation was noted at these temperatures showed the presence of R forms of the yeast.

In the case of *Zygosaccharomyces mandschuricus* both the R and G forms produced scum at all temperatures. At the two higher temperatures scum formation occurred in the S form of this yeast as in the other yeasts but here again, when cultures showing scum formation were plated and examined microscopically, they all showed the presence of the R forms.

As a result of these studies, it may be concluded that scum formation in yeasts occurs when the R form of the culture is present or in some cases when the G form is present.

Immunological Reactions of S, R and G forms

The S, R and G forms of the yeasts were grown on malt extract agar until abundant growth appeared. They were then suspended in saline washed, centrifugalized, and resuspended in saline. Preliminary experiments showed that it was necessary to use heavy suspensions for immunization. Therefore, a tube containing a heavy suspension of the yeasts was prepared as a standard. This turbidity standard was much more turbid than any of the standards used for bacterial antigens.

Rabbits were immunized against each of the three antigens by intravenous injections at three-day intervals. One cubic centimeter was given at the first injection and this amount was

TABLE 6

Showing the effect of temperature on scum formation of the various forms of yeasts

CULTURE	ONE WEEK		TWO WEEKS		THREE WEEKS		FOUR WEEKS	
	Malt broth	Plain broth	Malt broth	Plain broth	Malt broth	Plain broth	Malt broth	Plain broth
37°C.								
<i>S. cerevisiae</i> Saaz:								
S.	-	-	-	++	+	++	++	++
R.	++	++	++	++	++	++	++	++
G.	-	-	-	-	-	-	-	-
<i>S. cerevisiae</i> Froberg:								
S.	-	-	-	-	-	++	-	++
R.	++	++	++	++	++	++	++	++
G.	-	-	-	-	-	-	-	-
<i>S. ellipsoideus</i> :								
S.	-	-	-	-	-	++	-	++
R.	++	++	++	++	++	++	++	++
G.	-	-	-	-	-	-	-	-
<i>W. anomala</i> :								
S.	-	++	+	++	++	++	++	++
R.	++	++	++	++	++	++	++	++
G.	-	-	-	-	-	-	-	-
<i>Z. mandshuricus</i> :								
S.	-	-	-	-	-	++	-	++
R.	-	-	-	++	-	++	-	++
G.	-	++	-	++	-	++	-	++
29°C.								
<i>S. cerevisiae</i> Saaz:								
S.	-	+	-	++	+	++	-	++
R.	++	++	++	++	++	++	++	++
G.	-	-	-	-	-	-	-	-
<i>S. cerevisiae</i> Froberg:								
S.	-	-	-	-	-	-	-	-
R.	-	++	+	++	++	++	++	++
G.	-	-	-	-	-	-	-	-

++ = scum; + = ring; and - = negative.

S = smooth form; R = rough form; and G = gonidial form.

TABLE 6—Continued

CULTURE	ONE WEEK		TWO WEEKS		THREE WEEKS		FOUR WEEKS	
	Malt broth	Plain broth	Malt broth	Plain broth	Malt broth	Plain broth	Malt broth	Plain broth
29°C.—Concluded								
<i>S. ellipsoideus</i> :								
S	—	—	—	—	—	++	—	++
R	++	++	++	++	++	++	++	++
G	—	—	—	—	—	—	—	—
<i>W. anomala</i> :								
S	—	—	—	++	+	++	++	++
R	++	++	++	++	++	++	++	++
G	—	—	—	—	—	—	—	—
<i>Z. mandshuricus</i> :								
S	—	—	—	—	—	+	—	++
R	—	—	—	+	—	++	—	++
G	—	++	—	++	—	++	—	++
23°C.								
<i>S. cerevisiae</i> Saaz:								
S	—	—	—	—	+	—	+	—
R	++	++	++	++	++	++	++	++
G	—	—	—	—	—	—	—	—
<i>S. cerevisiae</i> Froberg:								
S	—	—	—	—	—	—	—	—
R	+	++	+	++	++	++	++	++
G	—	—	—	—	—	—	—	—
<i>S. ellipsoideus</i> :								
S	—	—	—	—	—	—	—	—
R	—	—	—	+	—	++	+	++
G	—	—	—	—	—	—	—	—
<i>W. anomala</i> :								
S	—	—	+	—	+	—	+	—
R	++	++	++	++	++	++	++	++
G	—	—	—	—	—	—	—	—
<i>Z. mandshuricus</i> :								
S	—	—	—	—	—	—	—	—
R	—	—	—	+	+	++	+	++
G	—	++	—	++	—	++	—	++

TABLE 6—Continued

CULTURE	ONE WEEK		TWO WEEKS		THREE WEEKS		FOUR WEEKS	
	Malt broth	Plain broth	Malt broth	Plain broth	Malt broth	Plain broth	Malt broth	Plain broth

17°C.

<i>S. cerevisiae</i> Saaz:								
S.....	-	-	-	+	-	+	-	+
R.....	++	++	++	++	++	++	++	++
G.....	-	-	-	-	-	-	-	-
<i>S. cerevisiae</i> Froberg:								
S.....	-	-	-	-	+	-	+	-
R.....	+	++	++	++	++	++	++	++
G.....	-	-	-	-	-	-	-	-
<i>S. ellipsoideus</i> :								
S.....	-	-	-	-	-	-	++	+
R.....	+	++	++	++	++	++	++	++
G.....	-	-	-	-	-	-	-	-
<i>W. anomala</i> :								
S.....	-	-	+	-	+	-	+	+
R.....	++	++	++	++	++	++	++	++
G.....	-	-	-	-	-	-	-	-
<i>Z. mandshuricus</i> :								
S.....	-	-	-	-	-	-	-	-
R.....	-	-	-	-	-	-	-	-
G.....	-	++	-	++	-	++	-	++

9°C.

<i>S. cerevisiae</i> Saaz:								
S.....	-	-	-	-	+	+	+	+
R.....	+	+	++	++	++	++	++	++
G.....	-	-	-	-	-	-	-	-
<i>S. cerevisiae</i> Froberg:								
S.....	-	-	-	-	-	-	-	-
R.....	+	+	++	++	++	++	++	++
G.....	-	-	-	-	-	-	-	-
<i>S. ellipsoideus</i> :								
S.....	-	-	-	-	-	-	-	-
R.....	-	-	-	-	-	-	-	-
G.....	-	-	-	-	-	-	-	-

TABLE 6—*Concluded*

CULTURE	ONE WEEK		TWO WEEKS		THREE WEEKS		FOUR WEEKS	
	Malt broth	Plain broth	Malt broth	Plain broth	Malt broth	Plain broth	Malt broth	Plain broth
9°C.— <i>Concluded</i>								
<i>W. anomala</i> :								
S.....	—	—	—	—	—	+	—	+
R.....	+	+	++	++	++	++	++	++
G.....	—	—	—	—	—	—	—	—
<i>Z. mandshuricus</i> :								
S.....	—	—	—	—	—	—	—	—
R.....	—	—	—	—	—	—	—	—
G.....	—	—	—	—	+	+	++	++

increased 1 cc. each time until five injections had been given. One week following the last injection of the antigen, trial titers were taken. If these were satisfactory, the animals were bled and the tests run; otherwise the injections were continued.

Agglutination and cross-agglutination tests were conducted employing antigens to which had been added sufficient phenol to

TABLE 7

Agglutination reactions of the S, R and G forms of yeasts

NAME OF ORGANISM	SERUM	ANTIGEN		
		S	R	G
<i>Saccharomyces cerevisiae</i> Saaz.....	S	1:1024	1:4	1:4
	R		1:256	1:16
	G		1:128	1:512

give a final concentration of 0.5 per cent. The results of these tests are given in table 7.

DISCUSSION

It has been possible to take single cell isolations of a pure culture of five species of yeasts and by subjecting them to the influence of certain chemical and physical agents to produce certain definite morphological, cultural, physiological and immunological variations. The new forms of the yeasts differ

wholly or partially from the form from which they were obtained. In changing from the original to the induced forms, they followed a certain definite sequence of changes which could be reproduced repeatedly and in an orderly manner. The various chemical and physical agents acted in essentially the same way and differed only in the degree and in the rapidity with which they caused the changes. The various forms when once obtained were in most cases stable and have been kept for several months with little or no tendency to revert to the original forms from which they were obtained.

It has been possible also, by certain definite procedures outlined, to convert the induced forms back to the original forms which, when obtained, had all of the characteristics of the original form.

The production of these various forms serves to clarify several points in connection with yeasts which have remained obscure or for which no lucid explanation has been given. Scum formation has long been one of the characteristics used in the identification of yeasts. Hansen in his numerous and valuable researches on yeasts observed in many species of yeasts such as *Saccharomyces validus*, *Saccharomyces intermedius*, *Saccharomyces pastorianus* and *Saccharomyces ellipsoideus* scum formation at the higher temperatures in a much shorter time than at the lower temperatures. For example, in the case of *Saccharomyces ellipsoideus* he made the following observation on the ability of this species of yeast to form scum at the following temperatures:

At 38°C.	no formation of scum
33-34°C.	sum formation complete at the end of 8-12 days
26-28°C.	sum formation complete at the end of 9-16 days
20-22°C.	sum formation complete at the end of 10-17 days
13-15°C.	sum formation complete at the end of 15-30 days
6- 7°C.	sum formation complete at the end of 2- 3 months
5°C.	no formation of scum

In the light of the work reported here (see table 6) and from Hansen's own observations, it is obvious that when this yeast was placed at the various temperatures, the tendency to scum formation was due to the dissociation of the S form of *Saccharomyces ellipsoideus* to the R form. Scum formation took place in a

shorter time at the higher temperatures than at the lower temperatures because the yeast dissociated more rapidly at these temperatures than at the lower temperatures. On the other hand the R form of the yeast formed scum at all temperatures while the S and G forms showed no scum formation at any of the temperatures. The one exception to this was the S form at 37°C. where scum was produced due to the dissociation of this form into the R form.

Under industrial conditions as in the manufacture of beer, a yeast which has given good results may suddenly give a beer with evident defects. Likewise, in the manufacture of industrial alcohol certain strains of yeasts which have been giving satisfactory yields may suddenly fail to produce satisfactory yields. In fact in some cases they fail to produce any alcohol.

A microscopic examination of the culture from the vats reveals small diplococci. In the past these have been regarded as bacterial contaminations. In the light of the present work it would appear that what has happened in such cases is that the yeasts have been transformed in the presence of the alcohol into the G form. The G form as the results here show is produced very abruptly from the S form in the presence of alcohol, within one week. Under the conditions found in an industrial alcohol plant where the yeasts were manufacturing their own alcohol the changes doubtless would be less abrupt.

Guilliermond (1919) states "Besides morphological variations, one may also observe physiological variations. A yeast may, for example, under certain conditions, induce more or less active fermentations in the same way that a certain bacterium, *Bacillus anthracis*, for instance, may be made avirulent; among the yeasts it is impossible to suppress the fermenting function. One may decrease it or even increase it but never entirely blot it out."

By "fermenting function" it is assumed that alcoholic fermentation is referred to. This work would indicate that it is possible entirely to suppress the ability of a yeast to produce alcohol by converting it into the G form. No G forms of any of the yeasts reported in this paper or any that are now being studied have produced an alcoholic fermentation. They produce an acid fermentation without the presence of gas.

Gelatin liquefaction is another instance where there is a fundamental difference between the various forms. The S and R forms do not liquefy gelatin while the G forms do. There is one exception to this in the case of the G form of *Saccharomyces cerevisiae* Saaz. None of the three forms of this yeast liquefied gelatin. It is obvious then that some of the aberrant results obtained in gelatin liquefaction may be explained on the basis of dissociation.

The immunological relationships are likewise interesting. The limited amount of work that has been done so far indicates a closer immunological relationship between the R and G forms than between the S form and the two other forms.

SUMMARY AND CONCLUSION

Dissociation was induced in cultures from single cell isolations of five different yeasts: viz., *Saccharomyces cerevisiae* Saaz, *Saccharomyces cerevisiae*, Froberg, *Saccharomyces ellipsoideus*, *Willia anomala*, and *Zygosaccharomyces mandshuricus*. Certain definite forms of these yeasts appeared constantly and have been designated as S, R, G, and T forms. The R, G, and T forms of the yeasts were induced from the normal S form by aging or serial transfer of the S form in the following media; lithium chloride broth and brilliant green broth; and by aging them in broths containing high concentrations of alcohol. They were also induced by desiccation and by abnormal temperatures. These forms have been described in detail in the paper.

The salient characteristics of the various forms are as follows: The S form of a yeast is the normal form described in the literature for most species and possesses the morphological, cultural, and physiological properties usually ascribed to the species. All the species reported here were sporogenic. The R form of a yeast consists of greatly elongated cells which form dull, rugose, wrinkled colonies having a filamentous edge when viewed under low-power magnification. This form likewise produces ascospores. The physiological properties of an R form are the same as those of an S form. Studies of scum formation at various temperatures revealed that the R form is a scum producer at all temperatures permitting growth; whereas, the S form produces scum only at those temperatures favorable for dissociation.

The G form of a yeast consists of cells greatly reduced in size from that of the S and R forms. These cells are asporogenic, produce an acid instead of an alcoholic fermentation and differ somewhat from the S and R forms in the sugars fermented. Upon the initial isolation, they grow very slowly on culture media, producing colonies microscopic in size at the end of a week's incubation. Upon becoming adapted to growth on culture media the G form usually produces a dull, thin, spreading colony.

A fourth type, the T form, is a transitional form of the yeast between the S or R form and the G form. It consists of highly refractile cells which produce the G form by the formation of a multitude of minute buds on the periphery of the cell. This form has never been cultured.

In the case of *Saccharomyces cerevisiae* Saaz, intermediate chromogenic forms have also been obtained: a pink S form which became mucoid in character upon being aged on an agar slant, and a black R form. Chromogenesis also occurred in one form of *Zygosaccharomyces mandshuricus*. The G form of this yeast produced bright orange-colored colonies.

Single cell isolations of the R and G forms of the yeasts have been changed to the normal S form by the use of a suitable technique.

Morphological, cultural, physiological and immunological studies were conducted on these induced forms of yeasts. From the results obtained, an attempt has been made to clarify some of the obscure points hitherto noted in the behavior of yeasts.

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PLATE 1

FIG. 1. Photomicrograph of S form of *Saccharomyces cerevisiae* Saaz. (450 \times)

FIG. 2. Photomicrograph of R form of *Saccharomyces cerevisiae* Saaz. (450 \times)

FIG. 3. Photomicrograph of G form of *Saccharomyces cerevisiae* Saaz. (450 \times)

FIG. 4. Photomicrograph of colony of S form of *Saccharomyces cerevisiae* Saaz. (100 \times)

FIG. 5. Photomicrograph of colony of R form of *Saccharomyces cerevisiae* Saaz. (100 \times)

FIG. 5A. Photomicrograph of colony of μ form intermediate between the S and R forms of *Saccharomyces cerevisiae* Saaz. (100 \times)

FIG. 6. Photomicrograph of colonies of G form of *Saccharomyces cerevisiae* Saaz. (100 \times)

FIG. 7. Photograph of colony of S form of *Saccharomyces cerevisiae* Saaz. Actual size.

FIG. 8. Photograph of colony of R form of *Saccharomyces cerevisiae* Saaz. Actual size.

FIG. 9. Photograph of colony of G form of *Saccharomyces cerevisiae* Saaz. Actual size. Note the presence of secondary colonies.

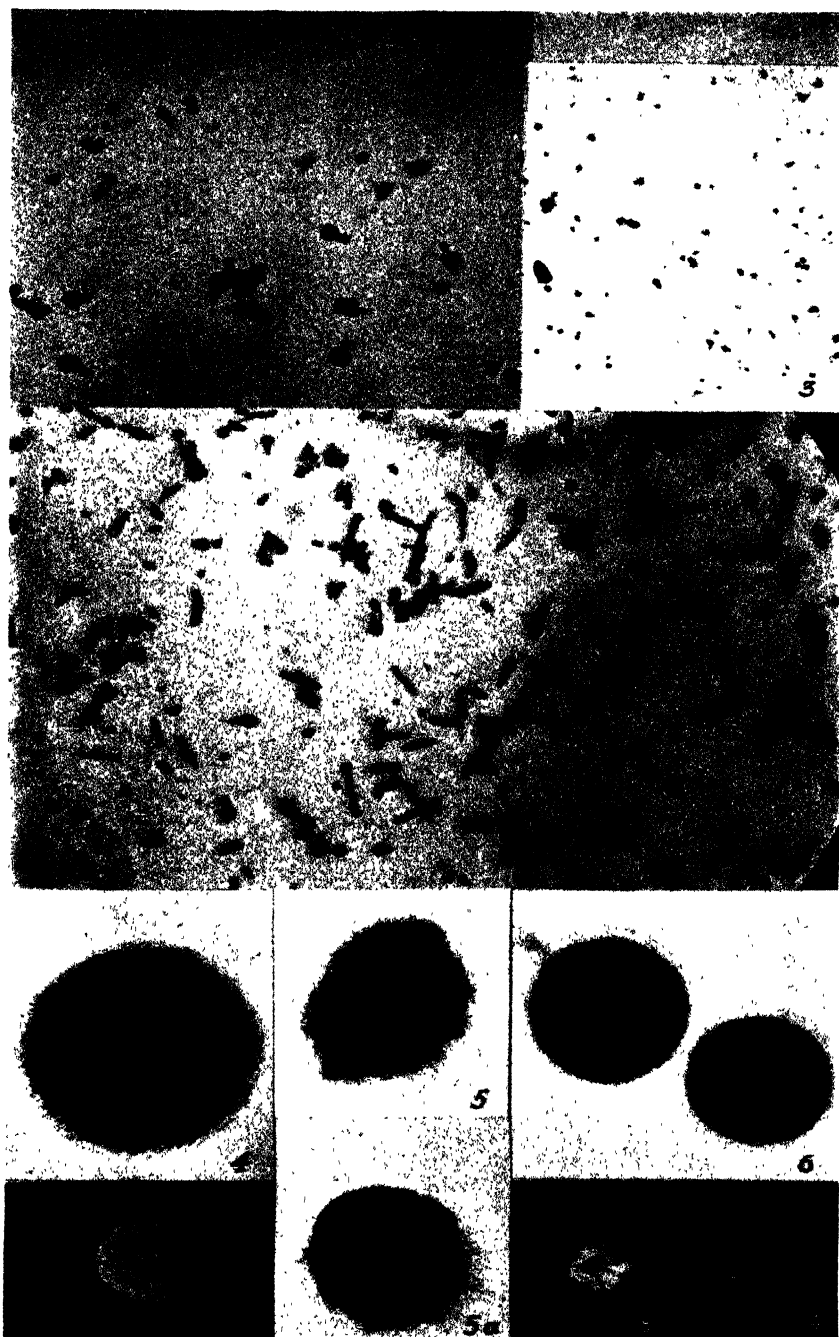


PLATE 2

FIG. 10. Photograph of slant cultures (from left to right) of the G, R and S forms of *Saccharomyces cerevisiae* Saaz.

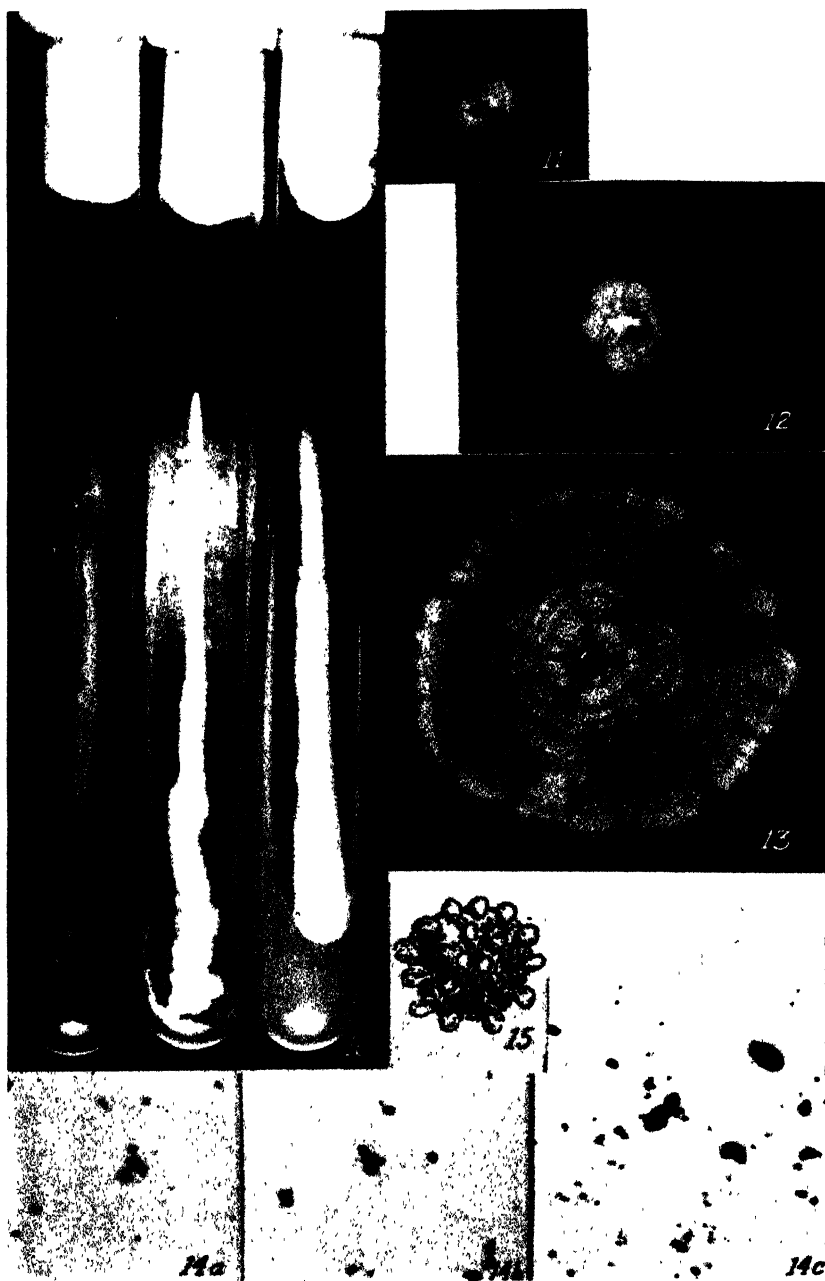
FIG. 11. Photograph of colony of S form of *Saccharomyces ellipsoideus*. Actual size.

FIG. 12. Photograph of colony of R form of *Saccharomyces ellipsoideus*. Actual size.

FIG. 13. Photograph of colony of G form of *Saccharomyces ellipsoideus*. Actual size.

FIG. 14. a, b, c Photomicrographs showing transitional forms of *Saccharomyces cerevisiae* Saaz. (450 \times)

FIG. 15. Sketch of transitional cell showing gonidia (2000 \times)



(F. W. Fabian and N. B. McCullough. Dissociation in yeasts.)

A NEW THERMOPHILIC ACTINOMYCES

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In the course of a study of the bacterial flora of various brands of pasteurized cheese, a thermophilic actinomyces was repeatedly isolated, Bernstein (1931). The organism occurred in smaller numbers than did the spore-forming rods, streptococci and micrococci. It attracted attention, however, by its ability to withstand a temperature of 140° to 160°F. for one-half hour, once, and possibly twice, during the course of the manufacture of the cheese. In view of the paucity of literature dealing with thermophilic actinomyces, a comprehensive study of its characteristics was undertaken. A summary of the work follows.

The mycelial filaments are about 0.5 to 0.7 μ in width and show a moderate degree of true branching. Lateral, terminal and intercalated chlamydospores are formed. The spirals described by Drechsler (1919) have not been observed. By methylene blue, the mycelium is stained very poorly; it retains the Gram stain, but is easily decolorized by treatment with alcohol; it stains very readily with carbol-fuchsin, but is non-acid-fast. Metachromatic granules are frequently demonstrated in the mycelium by either the Gram or carbol-fuchsin stain.

Growth occurs between pH 6.7 and 7.9, the optimum being 7.6. The optimum temperature for growth is 56°C. Colonies on agar are round, 1 cm. in diameter, flat, filamentous, mealy, with dull surface and fimbriate edge; odorless; friable and difficult to emulsify. There is a punched-out central portion, nearly colorless, and more transparent than the white peripheral zone. The medium is faintly etched. There is only slight growth in Czapek's broth, Waksman's (1919) modification. Maltose, mannitol, lactose, sucrose, glucose, dextrin, inulin, xylose and cellobiose

TABLE 1

ORGANISM	AUTHOR	GROWTH AT 37°C.	THERMAL DEATH POINT	OXYGEN RE- QUIRE- MENTS	COLOR	POTATO	MILK	BROTH	NT- TRACES	STARCH	GELATIN
<i>Cladosporium thermophilum</i>	Globig					Grows well					
<i>Thermomyces lanuginosus</i>	Kedrior	+	4 hours at 100°C.	Facultative anaerobe	Becomes green	Grows well					
	Tatkinaky	No growth under 42°C	1 minute at 100°C.		Darkens on standing		Acid coagulation and peptonisation				
<i>Thermosphaeromyces vulgaris</i>	Tatkinaky	No growth under 48°C.	20 minutes at 100°C.			Grows well	Acid coagulation and peptonisation				
	Tatkinaky		5 minutes at 100°C.	Facultative anaerobe							Not liquefied
<i>Streptothrix thermophila</i> , No. 12	Tatkinaky	+			Darkens on standing	Grows well					
<i>Streptothrix thermophila</i> , No. 20	Tatkinaky	No growth under 48°C.				Grows well	Not coagulated				Not liquefied
	Sames	+	10 seconds at 100°C.	Anaerobe, facultative aerobe	Yellowish grey	Grows well	Alkali and coagulation				
<i>Actinomyces thermophilus</i>	Gilbert	+			Grey turning dark	Grows well					
<i>Actinomyces thermophilus Bertheletii</i>	Miche*	+	10 minutes at 100°C.		Brown- yellow	Grows well					

<i>Thermomyces lanuginosus</i>	Mishe*	+	5 minutes at 100°C.	Grey-green	Grows well	Not coagulated	At bottom of tube	Reduced	Hydrolyzed	Not liquefied
<i>Actinomyces monosporus</i>	Schüttke	+		Grey-green	Grows well		Both on surface and at bottom of tube			Complete liquefaction
<i>Streptothrix</i> No. 8	Bruni	+		Chestnut color develops with time	Grows well					
<i>Streptothrix</i> No. 9	Bruni			Yellow	Grows well					
<i>Streptothrix</i> No. 12	Bruni									
<i>Actinomyces spinosporus</i> Spini	Velich									
<i>Actinomyces thermofastidiosus</i>	Bergey			Darkens on standing	Slight growth	No coagulation or peptonization	Colonies at bottom of tube	Reduced	Hydrolyzed	
<i>Actinomyces nonfastidiosus</i>	Bergey			Grey, turning dark	Grows well	Acid, coagulation, peptonization	Flocculent sediment	Reduced		
<i>Streptothrix thermophilus</i>	Eckford	No growth below 45°C.	15 minutes at 120°C.					Reduced	Hydrolyzed	
<i>Actinomyces thermophilus</i> Krohn	Krohn	Growth at 60° to 65°C		Brown	Grows well	Micro-aerophilic				
<i>Actinomyces casei</i>	Herau described	Growth at 40° to 60°C.	2 hours at 100°C.	White, medium unchanged	No growth	Coagulation and peptonization	Pellicle	Not reduced	Not hydrolyzed	

* Cited by Schüttke.

are not fermented. Indol is not formed. (Additional characteristics are listed in table 1.) The organism is non-pathogenic for rats and guinea pigs.

A review of the literature reveals that about 20 species of thermophilic actinomyces have been described. The table lists the organisms and the properties by which they may be differentiated. So far as can be determined by comparison of the listed characteristics, the organism which we have studied has not been previously encountered. It may, then, be regarded as a new species, for which the name *Actinomyces casei* is suggested.

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